Environmental effects of engineered nanomaterials

Estimations of Predicted No-Effect Concentrations (PNECs)

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Table of content

Preface

This report is part of the project "Nanomaterials – Occurrence and effects in the Danish Environment" ("NanoDEN"). NanoDEN was commissioned by the Danish EPA in December 2012 and runs until mid-2015 and is one among a number of projects funded by the Danish EPA on nanomaterials aiming to increase the knowledge and understanding regarding occurrence of engineered nanomaterials in Denmark and the risks posed by these to humans and the environment.

The NanoDEN project is part of the initiative of the Danish government and the Red-Green Alliance (a.k.a. Enhedslisten) called "Bedre styr på nanomaterialer" (Better control of nanomaterials) for 2012-2015 that focuses on the use of nanomaterials in products on the Danish market and their consequences for consumers and the environment.

The NanoDEN project is carried out by a project team with participation of COWI A/S (lead partner) (project leader: Jesper Kjølholt), the Technical University of Denmark (DTU Environment) (project leader: Anders Baun) and the Swiss Nano Modelling Consortium (SNMC) (project leader: Fadri Gottschalk).

DTU Environment is the lead institution for this report. The contributors to the report are Hans-Christian Holten Lützhøft, Nanna B. Hartmann and Anders Baun (DTU Environment), and Anna Brinch and Jesper Kjølholt (COWI A/S).
The authors would like to acknowledge Aiga Mackevica, Sara Nørgaard Sørensen, Lars Michael Skjolding and Rune Hjorth (DTU Environment) for their review of certain parts of Chapter 5.

A Steering Committee with the following participants was established for the NanoDEN project:

- Flemming Ingerslev, Danish EPA (Chairman and project responsible)
- Katrine Bom, Danish EPA
- Jørgen Larsen, Danish EPA
- Jesper Kjølholt, COWI A/S (project manager)
- Anders Baun, DTU Environment
- Fadri Gottschalk/Bernd Nowack, SNMC.

Executive summary

Background and Objective
The Danish EPA has under the Agreement "Better Control of Nanomaterials" (“Bedre styr på nanomaterialer”) launched a number of projects aiming at investigating and generating new knowledge on the presence of nanomaterials in products on the Danish market and assess the possible associated risks to consumers and the environment. One of the projects, NanoDEN, has the overall objective of assessing whether engineered nanomaterials (ENMs) give reason for concern in the Danish environment.

The current report covers subproject 5 of the NanoDEN project, which has the purpose of providing data on environmental effects of nine selected engineered nanomaterials (ENMs) in the form of Predicted No-effect Concentration (PNEC) values. Together with the subprojects on environmental fate modelling and exposure assessment, the results of this report will eventually feed into the concluding environmental risk assessment of the selected ENMs (subproject 6).

Selection of nanomaterials
In the context of this report ENMs are defined as manufactured materials with one or more external dimensions between 1 and 100 nm. In that respect ENMs is a part of a broader group of nanomaterials, which can result from natural, anthropogenic (incidental) and engineered (intentional) processes, however the NanoDEN project focuses on ENMs, as this is the most relevant group of nanomaterials from an up-stream regulatory point of view. The following materials have been selected for this project:

- Titanium Dioxide (TiO_2) (rutile and anatase)
- Zinc Oxide (ZnO)
- Silver (Ag)
- Carbon Nanotubes (CNTs)
- Copper Oxide (CuO)
- Nano Zero Valent Iron (nZVI)
- Cerium Dioxide (CeO_2)
- Quantum Dots (QDs)
- Carbon Black (CB)

The selection was based on expected production and use volumes in Denmark and their applications in relevant consumer products, industrial processes, and environmental remediation processes. In the NanoDEN project, $CuCO_3$ nanoparticles are included as case study material for copper-containing nanoparticles. However, in this report $CuCO_3$ has been replaced by CuO nanoparticles because CuO nanoparticles have been studied to some extent in ecotoxicity studies whereas studies for $CuCO_3$ are lacking. For copper-containing ENMs it has been found that the release of copper ions is of great importance in relation to the ecotoxicity of this metal and this is assumed to be a common feature of for both $CuCO_3$ and CuO.

Key findings
- Investigations have shown that currently accepted PNEC estimation approaches within the present European legislation (e.g. the legislation on chemicals, REACH) in principle can be used for nanomaterials as well. This concerns the assessment factor (AF) and species sensitivity distribution (SSD) approaches. These methods do, however, not take nano-

specific processes (such as aggregation) during the testing of nanomaterials into account and the tests may therefore not always be representative for natural conditions. Through a literature review carried out within the current project, three other methods were suggested: the probabilistic species sensitivity distribution (PSSD), the dissolved metal ion and the indicative no effect concentration (INEC).

- It was found that the current approach to select data for PNEC estimation favours effect studies conducted according to Good Laboratory Practice (GLP) and accepted guidelines. A consequence is that effect studies conducted according to guidelines for soluble chemicals may be unreliable as they do not take into account the specific nature of ENMs.

- Within the current project, an approach was therefore developed for transparent evaluation of the suitability of effect studies to test ENMs and thereby to what extent they are adequate for risk assessment. The approach has focused on nano-specific parameters and highlights knowledge gaps and limitations in relation to data availability and relevance.

- More than 1.200 scientific papers on effect studies of ENMs were found in the open literature. 500 of these revealed data on effects that potentially could be used for PNEC derivation. 50% of these studies used daphnia as the test organism, 30% used fish and 20% used algae. Hardly any chronic studies were performed with fish. Nevertheless, no single study obtained the best score for risk assessment adequacy.

- The number of sufficient effect studies adequate for risk assessment was, despite the large number of effect studies found, so low that PNEC estimation only could be made according to the AF approach.

- Using the available data silver nanoparticles were found to be the most toxic ENM (PNEC = 12 ng/L), while TiO_2 was found to be the least toxic (PNEC = 18 µg/L). Due to a lack of adequate data it was not possible to derive PNEC values for carbon black and quantum dots.

- The derived PNEC values in this report were generally on the same level or slightly lower than the PNEC values found in the open literature or in REACH registrations. Compared with ion and bulk PNEC values in REACH registrations, the derived nano PNEC values in this report were in same order of magnitude (silver) or one order of magnitude lower (titanium dioxide, zinc oxide and copper oxide).

On the following pages, the above mentioned key findings will be elaborated.

PNEC methodologies and applicability to ENMs

Two approaches for determining PNEC values for conventional soluble chemicals are recommended by REACH, namely: 1) the assessment factor (AF) approach and 2) the deterministic approach using species sensitivity distributions (SSDs) (ECHA, 2008). Both approaches use AFs (10-1,000 and 1-5, respectively) to cover uncertainties in the extrapolation, e.g. laboratory data to field impact. These approaches are in principle found to be applicable to ENMs as well in the REACH Implementation Project on Nanomaterials (Aitken, Bassan et al. 2011).

In the open literature another three approaches were found as alternatives for PNEC estimation of ENMs. The simplest is the Indicative No Effect Value (INEC), which, as the name says, is a value that is only indicative of the no-effect level. A more conservative approach applies the PNEC for the dissolved metal ion divided by a factor of two in order to take into account any nano-specific effects, without at all addressing these effects. In the cases with sufficient data, the currently applied methods will be more suitable. Closely related to the SSD is the probability species sensitivity distribution (PSSD), which considers both acute and chronic EC_X and NOEC data. AFs are used to

produce long-term NOECs with the aim of performing the probability modelling. It was in fact found that the PSSD approach resulted in PNEC values in the same order of magnitude and within a factor of 4 (titanium dioxide, zinc oxide, silver, copper oxide and cerium dioxide), as PNEC values derived in accordance with the AF approach. Only the PNEC value for CNTs showed to be to orders of magnitude larger by applying the PSSD approach compared with the AF approach. For all methods solid underlying effect data are required to produce a reliable PNEC. However, for the PSSD method this is most likely more pronounced, because the current effect data in the literature are seriously influenced by studies not suited for ENMs (see later), as all available effect data are included in the modelling. At present the AF approach is the most widely used and for the time being, there are no nano-specific arguments that this approach should not work, except for the fact that ENMs agglomerate having serious impact on factors like dilution, i.e. application of AFs. Application of a large AF may potentially bring a NOEC into a concentration level where the agglomeration is less pronounced leaving more of the active nanoparticle in its free state.

It is, however, of utmost importance to emphasize that the validity of the assumption, that PNEC values for ENMs can be estimated as though they were dissolved chemicals, has not been addressed. Given the range of nano-specific concerns listed in this report, it is at present not possible to claim that the use of the current approaches ensure that organisms will be protected at concentrations below the derived PNEC. In other words, specific circumstances related to ENMs, which differ from conventional soluble chemicals, could likely affect the validity of the approach for deriving PNEC values in an unpredictable manner.

Data selection for PNEC estimation

In order to derive PNEC values according to the approaches currently recommended by the European Chemicals Agency (ECHA), the regulatory relevance and reliability of a study needs to be evaluated. In the ECHA guidance document (ECHA 2011) it is recommended that the risk assessment adequacies are assessed according to the Klimisch score where studies conducted according to GLP and accepted guidelines are preferred. At present there are no guidelines for effect studies of ENMs. This often results in that ENMs are tested according to guidelines for conventional soluble chemicals, which can lead to imprecise test results. It has therefore also been suggested that other endpoints and/or test organisms may be more relevant. Seen in this light, data generated by non-standard tests, but with emphasis on particular ENMs, could improve the scientific basis of risk assessment. However, it is acknowledged that some criteria for data quality and comparability are needed.

For the purpose of this work, a scoring system was developed for assessing the regulatory adequacy of ecotoxicological studies of ENMs for PNEC estimation. The system assesses the effect studies in two dimensions, so that both reliability and relevance are assessed. The study reliability is assessed according to a list with 21 criteria focusing on the characterisation and exposure assessment of the ENM and the relevance is assessed according to a list with 13 criteria. For both dimensions, each criterion is assessed on a scale from 0-3 and each criterion is assigned a weight from 1-3 (see Figure 1). Effect studies assessed within the white or grey area of the graph were included in the PNEC determination, whereas effect studies within the dark grey area were not included in the PNEC determination.

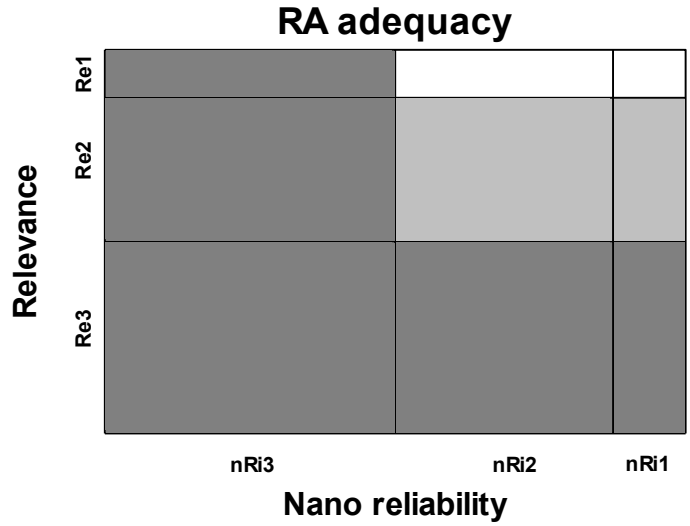


Figure 1 – Overview of the developed two-dimensional approach developed for evaluation of the risk assessment adequacy of effect studies. White area represents studies "adequate for use for regulatory purposes", grey area represents studies "may be adequate for use for regulatory purposes" and dark grey area represents studies "not adequate for use for regulatory purposes".

PNEC values for the ion, bulk and nano-forms of the case study ENMs

When the compiled ENM effect studies were assessed according to the developed approach, a range of the studies were found adequate for risk assessment and thereby adequate for deriving PNEC values, see right column of Table 1. PNEC values found in the open scientific literature, as well as PNEC values for the ion-, bulk and nano-forms from ECHA, are also shown in Table 1.

Table 1 - Overview of $PNEC_{freshwater}$ values for different forms of the nanomaterials in this report. All values are in µg/L.

	ECHA			Scientific literature		
Material	**ion***	**bulk***	**nano***	**AF approach§**	**PSSD approach§**	**This report#**
TiO_2	-	238	-	1-5.8	61	18
ZnO	20.6	20.6	-	0.042-2,194	9.9	2.5
Ag	0.04	0.04	-	0.001-1	0.01	0.012
CNTs	-	-	430/780	40	60	0.84
CuO	7.8	7.8	-	-	0.48	0.34
nZVI	-	-	-	-	-	5
CeO_2	-	-	-	52-108	2	5.2
QDs	-	-	-	-	-	-
CB	-	-	5,000/50,000	-	-	-

-: indicates that no data was identified or that PNEC values could not be established at present;

*: For "ion" and "bulk" the SSD approach with AFs of 1-3 were used, except for bulk TiO_2 where the AF approach was used applying an AF of 100, for "nano" the AF approach was used applying an AF of 10 for CNTs and 1,000 and 100 for CB, respectively;

§: AF: AF approach applying an AF of 1,000 except for CeO_2, where an AF of 50 was applied and PSSD: PSSD approach where varying AFs are used to derive NOEC values from EC_X values;

#: PNEC values based on the AF approach applying an AF of 50 (except for Ag, CuO and nZVI, where 100 was used).

NOTE: The PNEC estimations are based on the assumptions that 1) the current test methods for soluble chemicals are applicable to nanomaterials as well, and 2) that the current extrapolation methods are valid for nanomaterials. Both of these assumptions are highly questionable and have not been validated. The values should therefore be taken as indicative for the order of magnitude for PNEC given the current regulatory recommendations for PNEC estimation and not be used as the definitive protective concentration for the environment.

It is seen that the PNEC values span several orders of magnitude, depending on the ENM, with silver nanoparticles being the most toxic with a PNEC value of 12 ng/L and titanium dioxide the least toxic with a PNEC value of 18 µg/L.

Given the reservations presented in this report[1], an AF of 50 was applied for the PNEC estimation for all ENMs (except for Ag, CuO and nZVI where AFs of 100 were used). When comparing the values listed in Table 1 for the bulk, ion, and nano-forms of the ENMs it is evident that the PNEC values for CuO-NPs are around 23 times lower than the corresponding PNEC for the ion and bulk forms of Cu. For TiO_2-NPs, ZnO-NPs and Ag-NPs the corresponding differences are 13, eight and three times, respectively, i.e. either one order of magnitude lower (CuO, TiO2 and ZnO) or in the same order of magnitude (Ag). To what extent this reflects the ion dissolution, dose metric or exposure quantification is not possible to verify within the current time and knowledge base.

Comparing the PNEC values derived in this report with PNEC values (AF approach) found in the open scientific literature, it is seen that overall the PNEC values are in the same order of magnitude. However, some are lower and some are higher. Comparing with the PNEC values determined using the PSSD approach all PNEC values are found in the same order of magnitude, except for CNTs which is different by two orders of magnitude. In relation to this it must be stressed that CNTs are genuinely new materials for which comparable larger or smaller sizes exist.

Considering the relatively low AF of 50 used for the PNEC estimations of most ENMs in this report, it does not seem to be the AF, and thereby the lack of data, that is causing these PNEC values for ENMs to be lower both in comparison to other forms of the materials and to PNEC values from the literature.

Data availability, gaps and uncertainty

In the light of this report's analysis of gaps and uncertainties for each of the studied case ENMs and on the basis of articles found in the open scientific literature, a number of general gaps and uncertainties with respect to establishment of PNEC values for ENMs can be identified:

1. *Limited number of studies at different trophic levels*: It is generally found that the diversity of organisms tested is very low with respect to the trophic level, resulting in use of the AF approach alone for PNEC estimation.

2. *Lack of studies from different environmental compartments*: For all of the ENMs included in this report there is a pronounced lack of studies from other environmental compartments than freshwater. For conventional soluble chemicals there is an option of extrapolation from freshwater tests to other compartments by the use of partitioning coefficients. However, interpolation between compartments is not possible for ENMs. Thus, this data gap can only be closed by additional testing in the compartments at question, e.g. marine water and wastewater treatment plants.

[1] i.e. sometimes the chronic data are not fulfilling requirements, e.g. using LOEC or significant effect as a substitute for NOEC or sometimes acute data were not fulfilling requirements, e.g. not using the prescribed test duration or reporting NOEC instead of EC_{50}.

3. *Most studies focused on acute toxicity*: It was revealed from literature that the vast majority of ecotoxicity data are short-term tests. For the ENMs for which it was possible to establish a PNEC value, results from chronic tests were typically available from only two studies at two different trophic levels, i.e. algae and daphnia, resulting in the use of 50 as the AF. To obtain less uncertainty in the determination of PNEC in chemical safety assessment more studies focussed at chronic endpoints are needed.

4. *Most studies focused on zooplankton:* In association with the previous point, the far majority of effect studies were focused on testing daphnia (50%) in comparison to algae (20%) and fish (30%). Hardly any chronic fish studies were performed, possibly due to the high expenses and ethical considerations associated with fish studies.

5. *Testing of high ENM concentrations*: Often high and environmentally unrealistic concentrations are used in standardized ecotoxicity tests influencing the ENM behaviour and bioavailability. This may lead to either false-negative or false-positive results, which may influence the validity of the PNEC estimation.

The quality of published data is crucial for the process of risk assessment. This is true for both conventional and alternative approaches to PNEC estimation and risk characterisation. As shown through the literature review in this report there are challenges and obvious problems regarding the current framework for deriving PNEC values: 1) that effect studies are based on guidelines developed for soluble chemicals and therefore not suitable for nanomaterials and 2) that effect studies are assessed for their risk assessment adequacy according to the Klimisch score, which by nature favours studies conducted according to GLP and in accordance with current guidelines.

Regarding the estimation of PNEC values for nanomaterials the primary problem is that most of the available effect data are not reliable and there is as such a lack of valid data adequate for risk assessment. When effect studies are conducted according to currently accepted international (modified) guidelines developed for soluble chemicals, the inherent properties of the tested ENM will often be reason for varying exposure conditions during the ecotoxicological testing. This may in the end result in misleading test results.

This report's specific recommendation is therefore that PNEC estimations of nanomaterials are conducted after the effect studies have been assessed for their risk assessment adequacies according to the transparent and reproducible approach developed, documented and applied in this report.

Dansk sammendrag

Baggrund og mål
Den danske Miljøstyrelse har som led i udmøntningen af aftalen "Bedre styr på nanomaterialer" igangsat en række projekter, der sigter mod at undersøge og skabe ny viden om tilstedeværelsen af nanomaterialer i produkter på det danske marked og vurdere eventuelle risici i forhold til forbrugerne og miljøet. Et af projekterne, NanoDEN, har det overordnede formål at vurdere, om industrielt fremstillede nanomaterialer (ENMs) giver grund til bekymring i det danske miljø.

Nærværende rapport beskriver delprojekt 5 i NanoDEN projektet der har til formål at levere data for miljøeffekter af ni udvalgte ENMs i form af Predicted No-Effect Concentration (PNEC) værdier. Dette vil, sammen med delprojekterne om modellering af skæbne og eksponering i miljøet, i den sidste ende indgå i den efterfølgende miljørisikovurderingen af de valgte ENMs (delprojekt 6).

Udvælgelse af nanomaterialer
I forbindelse med denne rapport defineres ENMs som materialer fremstillet med en eller flere eksterne dimensioner på mellem 1 og 100 nm. I den henseende er ENMs en del af en bredere gruppe af nanomaterialer, der kan forårsages af naturlige, menneskeskabte (tilfældige) og forarbejdede (forsætlige) processer, men NanoDEN projektet har fokus på ENMs, da dette er den mest relevante gruppe af nanomaterialer fra et opstrøms lovgivningsmæssigt synspunkt. Følgende materialer er valgt til dette projekt:

- Sølv (Ag)
- Titaniumdioxid (TiO_2) (rutil og anatase)
- Zinkoxid (ZnO)
- Kulstofnanorør (CNTs)
- Kobberoxide (CuO)
- Nanojern i oxidationstrin nul (nZVI)
- Ceriumdioxid (CeO_2)
- Carbon black (CB)
- Kvantepunkter (QDs)

Udvælgelsen af disse nanomaterialer er baseret på forventning om høje produktions- og anvendelsesmængder i Danmark samt på forventning om anvendelser i relevante forbrugerprodukter, industrielle processer og miljøteknisk remediering. I NanoDEN projektet indgår $CuCO_3$ nanopartikler som casemateriale for kobberholdige nanopartikler. I denne rapport er $CuCO_3$ dog blevet erstattet af CuO nanopartikler på grund af, at CuO nanopartikler i langt større grad er undersøgt for dets økotoksiske effekter, hvorimod undersøgelser for $CuCO_3$ mangler. For kobberholdige ENMs er frigivelsen af kobberioner fundet at være af stor betydning for økotoksiciteten af dette metal, og dette formodes at gælde for såvel $CuCO_3$ som for CuO.

De vigtigste resultater
- Undersøgelser har vist, at de aktuelt accepterede metoder til estimering af PNEC i den nuværende EU-regulering (bl.a. kemikaliereguleringen, REACH) i princippet også kan bruges til nanomaterialer. Det drejer sig om ”assessment factor” (AF) og ”species sensitivity distribution” (SSD) metoderne. Imidlertid tager metoderne ikke direkte hensyn til nano-specifikke processer (eksempelvis agglomerering) under testning og er således ikke altid

repræsentative for naturlige forhold. I nærværende projekt viste litteraturgennemgangen at der er foreslået yderligere tre metoder til PNEC bestemmelse for ENMs: "probabilistic species sensitivity distribution" (PSSD), "dissolved metal ion" (opløst metalion) og "indicative no effect concentration" (den vejledende nuleffekt koncentration).

- Det viste sig, at den nuværende metode til valg af data til PNEC bestemmelse favoriserer effektundersøgelser udført i henhold til GLP (good laboratory practice) og accepterede testguidelines. En konsekvens er, at effektstudier udført i overensstemmelse med retningslinjer for opløselige kemikalier ikke altid er pålidelige, fordi de ikke tager hensyn til nanomaterialers særlige egenskaber.

- Derfor er der i dette projekt udviklet og anvendt en metode til transparent evaluering af effektstudiers egnethed til testning af ENMs og dermed i hvilken grad de er egnede til risikovurdering. Metoden har fokus på nano-specifikke parametre og fremhæver endvidere mangler og begrænsninger i viden i forhold til tilgængeligheden af data og deres relevans.

- I den åbne litteratur blev der fundet mere end 1.200 videnskabelige artikler med referencer til effektstudier af ENMs. 500 af disse viste data for effekter, der potentielt kan anvendes til PNEC bestemmelse. 50% af disse undersøgelser anvendte dafnier som testorganisme, 30% anvendte fisk og 20% brugte alger. Der var stort set ingen kroniske undersøgelser med fisk. Dog var der intet studie der opnåede den bedste score for egnethed til risikovurdering.

- På trods af at der blev fundet mange effektstudier, var der så få risikovurderingsegnede studier, at PNEC kun kunne udledes ved brug af AF-metoden.

- Ved at bruge de tilgængelige data, blev sølvnanopartikler fundet som det mest giftige ENM (PNEC = 12 ng/L), mens TiO_2 viste sig at være mindst giftigt (PNEC = 18 ug/L). På grund af datamangel var det ikke muligt at udlede PNEC-værdier for carbon black og kvantepunkter.

- De PNEC værdier, der blev bestemt i denne rapport, var generelt på samme niveau eller lidt lavere end PNEC værdier fundet i den åbne litteratur eller i REACH registreringer. Sammenlignet med ion og bulk PNEC-værdier i REACH registreringerne, var PNEC-værdierne bestemt i denne rapport i samme størrelsesorden (sølv) eller en størrelsesorden lavere (titaniumdioxid, zinkoxid og kobberoxid).

På de følgende sider vil de vigtigste resultater blive uddybet.

PNEC metoder og deres anvendelighed overfor ENMs

REACH anbefaler to metoder til bestemmelse af PNEC-værdier for de traditionelt opløselige kemikalier, nemlig: 1) "assessment factor" (AF) og 2) "species sensitivity distribution" (SSD) metoderne (ECHA, 2008). Begge metoder bruger usikkerhedsfaktorer (henholdsvis 10-1.000 og 1-5) for at dække usikkerheder i ekstrapolation, eksempelvis fra laboratorie til miljø. Disse metoder er i henhold til REACH implementeringsprojektet for ENMs (RIPoN3) i princippet også gældende for ENMs (Aitken, Bassan et al. 2011).

I den åbne litteratur findes yderligere tre metoder som alternativer til PNEC bestemmelse af ENMs. Det enkleste er "den vejledende nuleffekt koncentration", der, som navnet siger, er en værdi, der kun er vejledende for niveauet for nuleffekt. En mere konservativ tilgangsvinkel er, at halvere PNEC for den opløste metalion, for at tage hensyn til eventuelle nanospecifikke effekter men siger i sig selv intet om disse effekter. I de tilfælde hvor der er tilstrækkelige data vil de aktuelt anvendte metoder derfor være bedre. Den tredje metode, der bygger på SSD-metoden, er "probabilistic species sensitivity distribution" (PSSD), som inddrager både akutte og kroniske EC_X og NOEC data. Der anvendes usikkerhedsfaktorer for at estimere kroniske NOEC værdier, der sidenhen bruges til at

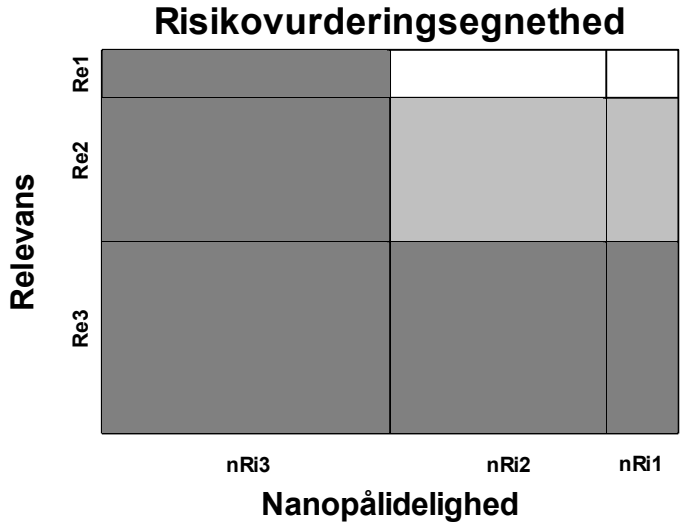
udføre en sandsynlighedsmodellering. PSSD-metoden, på den anden side, viste sig rent faktisk at give PNEC-værdier i samme størrelsesorden og inden for en faktor 4 (titaniumdioxid, zinkoxid, sølv, kobberoxid og ceriumdioxid), som PNEC-værdier udledt i henhold til AF-metoden. Kun PNEC for kulstofnanorør viste sig at være to størrelsesordener større ved brug af PSSD i forhold til AF-metoden. For alle metoderne er det under alle omstændigheder nødvendigt med valide underliggende effektdata, for at kunne beregne en pålidelig PNEC. Dog er det for PSSD metoden endnu mere nødvendigt, da de aktuelle effektdata i litteraturen er alvorligt påvirket af brugen af testmetoder, der ikke egner sig til test af ENMs (se senere), idet alle tilgængelige data inddrages i modelleringen. På nuværende tidspunkt er AF-metoden den mest udbredte og indtil videre er der ingen nano-specifikke argumenter for, at denne fremgangsmåde ikke bør anvendes. Dette såfremt der ses bort fra at ENMs agglomererer, og at det har alvorlige konsekvenser for faktorer som fortynding, dvs. anvendelse af usikkerhedsfaktorer. Ved anvendelsen af en høj usikkerhedsfaktor kan NOEC potentielt bringes ned i et koncentrationsniveau, hvor agglomereringen er mindre udtalt og dermed vil efterlade flere af de aktive nanopartikler i fri tilstand.

Det skal dog understreges, at det endnu ikke er undersøgt om det er gyldigt at antage at PNEC-værdier for ENMs kan bestemmes med samme principper som for opløste kemikalier. I betragtning af den række af nano-specifikke problemer, der er anført i denne rapport, kan det i skrivende stund ikke hævdes, at de nuværende metoder sikrer beskyttelse af miljøet ved koncentrationer under den beregnede PNEC. Der er med andre ord, særlige omstændigheder for ENMs, der adskiller sig så betydeligt fra traditionelle opløselige kemikalier, at der er usikkerhed om gyldigheden af de eksisterende metoder til PNEC bestemmelse.

Valg af data til PNEC estimering

For at kunne bestemme PNEC i henhold til de metoder der i øjeblikket er anbefalet af EU's kemikalieagentur (ECHA), skal effektstudiets pålidelighed og relevans evalueres. I vejledningen fra ECHA (ECHA 2011) anbefales det at effektstudiernes risikovurderingsegnethed evalueres i henhold til Klimischskalaen, der lægger vægt på studier udført efter GLP og anerkendte guidelines. I øjeblikket mangler der retningslinjer for effektstudier af ENMs, hvilket resulterer i, at effektstudier for ENMs ofte testes i henhold til retningslinjer for opløselige kemikalier. Dette kan føre til unøjagtige testresultater. Det er derfor også blevet foreslået, at andre end-points og/eller testorganismer kan være mere relevante. Set i det lys, kan data genereret af ikke-standardiserede tests, men med fokus på det enkelte ENM, forbedre det videnskabelige grundlag for risikovurdering. Dog er visse kriterier for datakvalitet og sammenlignelighed en nødvendighed.

Med henblik på dette arbejde blev et pointsystem udviklet til at evaluere ENM effektstudiers risikovurderingsmæssige egnethed til PNEC bestemmelse. Systemet vurderer studierne i to dimensioner, således at både pålidelighed og relevans evalueres. Studiets pålidelighed evalueres i forhold til en liste med 21 kriterier med fokus på karakteriseringen og eksponeringen af nanomaterialet og relevansen vurderes ud fra en liste med 13 kriterier. For begge dimensioner gælder at hvert enkelte kriterie vurderes på en skala fra 0-3 og at hvert enkelt kriterie kan vægtes fra 1-3 (se Figur 1). Effektstudier med vurderinger i det hvide eller grå område af grafen, blev herefter taget i betragtning til PNEC bestemmelse, hvor effektstudier i det mørkegrå område, ikke blev taget i betragtning til PNEC bestemmelse.



Figur 1 – Overblik over den udviklede todimensionelle metode til evaluering af et effektstudies risikovurderingsegnethed. Det hvide område repræsenterer studier der anses for "tilstrækkelige" i lovgivningsøjemed, det grå område repræsenterer studier der "kan være tilstrækkelige" og det mørkegrå område repræsenterer studier der "ikke er tilstrækkelige" i lovgivningsøjemed.

PNEC-værdier for ion-, bulk- og nanoformerne af de undersøgte ENMs

Efter at have vurderet de fundne ENM effektstudier i henhold til det udviklede system, blev en række af studierne fundet risikovurderingsmæssigt egnede til at bruge til PNEC-bestemmelse, se højre kolonne i Tabel 1. I Tabel 1 er ligeledes angivet PNEC-værdier fundet i den videnskabelige litteratur, samt PNEC-værdier for ion-, bulk- og nanoformerne fra ECHA.

Tabel 1 – Overblik over ferskvands PNEC værdierne for de forskellige nanomaterialer udvalgt i denne rapport. Alle værdier er i µg/L.

	ECHA			Videnskabelig litteratur		
Materiale	**ion***	**bulk***	**nano***	**AF metode§**	**PSSD metode§**	**Denne rapport#**
TiO_2	-	238	-	1-5,8	61	18
ZnO	20,6	20,6	-	0,042-2.194	9,9	2,5
Ag	0,04	0,04	-	0,001-1	0,01	0,012
CNTs	-	-	430/780	40	60	0,84
CuO	7,8	7,8	-	-	0,48	0,34
nZVI	-	-	-	-	-	5
CeO_2	-	-	-	52-108	2	5,2
QDs	-	-	-	-	-	-
CB	-	-	5.000/50.000	-	-	-

-: indikerer at ingen data kunne findes eller at PNEC ikke kan bestemmes på nuværende tidspunkt;

*: For "ion" og "bulk" blev SSD metoden og usikkerhedsfaktorer på 1-3 anvendt, bortset fra bulk TiO_2, hvor AF metoden blev anvendt med en usikkerhedsfaktor på 100, for "nano" blev AF metoden og en usikkerhedsfaktor på 10 for CNTs og henholdsvis 1.000 og 100 for CB;

§: AF: AF metoden ved brug af en usikkerhedsfaktor på 1.000, bortset fra CeO_2, hvor en usikkerhedsfaktor på 50 blev anvendt og PSSD: PSSD metoden ved brug af forskellige usikkerhedsfaktorer til at udlede NOEC værdier fra EC_X værdier;

#: PNEC værdier bestemt ved brug af AF metoden med en usikkerhedsfaktor på 50 (bortset fra Ag, CuO og nZVI, hvor 100 blev brugt).

BEMÆRK: PNEC-bestemmelserne er baseret på følgende antagelser at, 1) de nuværende testmetoder for opløselige kemikalier også gælder for nanomaterialer, og 2) de nuværende ekstrapoleringsmetoder er gældende for nanomaterialer. Begge disse antagelser er yderst tvivlsomme og ikke validerede. PNEC-bestemmelsen skal derfor tages som vejledende for størrelsesorden for PNEC-værdierne, i betragtning af de nuværende lovgivningsmæssige anbefalinger til PNEC estimering og dermed ikke anvendes som den endelige beskyttende koncentration for miljøet.

Det ses, at PNEC-værdierne strækker sig over flere størrelsesordener, afhængigt af det enkelte ENM med sølvnanopartikler som de mest giftige med en PNEC-værdi på 12 ng/L, og titaniumdioxid som de mindst giftigt med en PNEC-værdi på 18 µg/L.

I betragtning af de forbehold, der præsenteres i denne rapport[2], blev en usikkerhedsfaktor på 50 anvendt til PNEC bestemmelse for alle ENMs (bortset fra Ag, CuO og nZVI hvor en usikkerhedsfaktor på 100 blev anvendt). Når man sammenligner PNEC værdierne for ion-, bulk- og nanoformerne anført i Tabel 1, ses det at PNEC-værdierne for CuO-NPs er 23 gange lavere end de tilsvarende PNEC-værdier for ion- og bulkformer af Cu. For TiO_2-NPs, ZnO-NPs og Ag-NPs er de tilsvarende forskelle henholdsvis 13, otte og tre gange. Således enten en størrelsesorden lavere (CuO, TiO2 og ZnO) eller i samme størrelsesorden (Ag). Det er ikke muligt med den eksisterende viden at afgøre om det afspejler frigivelse af metalioner, om det er enheden man bruger til at dosere eller om det er grundet problemer med at kvantificere eksponeringen.

Sammenligner man med PNEC-værdierne udledt i denne rapport med PNEC-værdier (AF-metode) fundet i litteraturen, ses overordnet PNEC-værdier i samme størrelsesorden, dog med forskelle både til den lave og den høje side. Sammenligner man med PNEC-værdierne bestemt med PSSD-metoden ses alle PNEC-værdier at være i samme størrelsesorden, dog bortset fra CNTs med en forskel på to størrelsesordner. I denne sammenhæng skal det nævnes, at CNTs er genuine nye materialer, for hvilke der hverken findes sammenlignelige større eller mindre former.

Men i betragtning af den relativt lave usikkerhedsfaktor på 50 der er anvendt til PNEC bestemmelse i denne rapport, synes det ikke at være usikkerhedsfaktoren og dermed manglen på data, der er årsag til disse lavere PNEC-værdier for ENMs både i forhold til de andre materialeformer og PNEC-værdier fra litteraturen.

Datatilgængelighed, mangler og usikkerhed
På baggrund af denne rapports analyse af mangler og usikkerheder for hvert af de undersøgte ENMs, samt fra artikler i litteraturen, er en række generelle mangler og usikkerheder med hensyn til PNEC-bestemmelse for ENMs blevet identificeret:

1. *Begrænset antal undersøgelser på forskellige trofiske niveauer*: Det er generelt fundet, at der kun er testet få arter på hvert trofisk niveau, hvilket resulterer i at kun AF-metoden kan bruges til PNEC-bestemmelse.

2. *Mangel på effektstudier i forskellige miljømatricer*: For alle ENMs medtaget i denne rapport, er der en udtalt mangel på undersøgelser fra andre miljømatricer end ferskvand. For traditionelle opløselige kemikalier er det en mulighed at ekstrapolere fra ferskvandstests til andre matricer ved brug af fordelingskoefficienter. Men ekstrapolation fra ferskvand til andre matricer er ikke muligt for ENMs. Således kan denne mangel på data kun udfyldes med yderligere forsøg i de pågældende matricer, eksempelvis havvand og renseanlæg.

[2] at enkelte kroniske data undertiden ikke opfylder kravene til datakvalitet, eksempelvis ved at bruge LOEC eller en signifikant effekt som erstatning for NOEC eller at enkelte akutte data undertiden ikke opfylder kravene, eksempelvis ved ikke at bruge den foreskrevne testvarighed eller ved at rapportere NOEC i stedet for EC_{50}.

3. *De fleste undersøgelser har haft akut toksicitet i fokus*: Litteraturundersøgelsen viste at langt størstedelen af de fundne økotoksicitetsdata er kortvarige test. For de ENMs hvor det var muligt at bestemme en PNEC-værdi, var der typisk kun kroniske resultater tilgængelige fra to studier på to forskellige trofiske niveauer, dvs. alger og dafnier, hvilket resulterede i brugen af 50 som usikkerhedsfaktor. For at opnå mindre usikkerhed i PNEC bestemmelsen i kemisk sikkerhedsvurdering, er der behov for flere undersøgelser med fokus på kroniske end-points.

4. *De fleste undersøgelser fokuserede på zooplankton*: I forbindelse med det foregående punkt, fokuserede langt størstedelen af effektstudierne på at teste dafnier (50%) sammenlignet med alger (20%) og fisk (30%). Stort set ingen kroniske fiskestudier blev fundet, muligvis på grund af de høje omkostninger samt etiske overvejelser, der er forbundet med fiskestudier.

5. *Test af ENMs i høje koncentrationer*: Ofte bliver høje og miljømæssigt urealistiske koncentrationer anvendt i standard økotoksicitetsstudier, hvilket påvirker hvordan det enkelte ENM opfører sig i testen og om det er biotilgængeligt. Dette kan føre til enten falsk negative eller falsk positive resultater, hvilket igen kan påvirke PNEC-bestemmelsens validitet.

Kvaliteten af de publicerede data er altafgørende for risikovurderingen. Dette gælder for både konventionelle og alternative metoder til PNEC-bestemmelse og risikokarakterisering. Litteraturgennemgangen i denne rapport viste, at der er udfordringer og åbenlyse problemer med de nuværende rammer for PNEC-bestemmelse, nemlig at 1) effektstudier er baseret på testguidelines udarbejdet for opløselige kemikalier og derfor ikke er egnede til ENMs og 2) effektstudier vurderes for deres risikovurderingsegnethed i henhold til Klimisch skalaen, der i sagens natur favoriserer undersøgelser udført i henhold til GLP og i overensstemmelse med nuværende testguidelines.

Med hensyn til bestemmelse af PNEC-værdier for nanomaterialer, er problemet primært, at de fleste effektdata der kan findes generelt ikke er pålidelige og at der således mangler valide data, der er egnede til risikovurdering. Når effektstudier udføres i overensstemmelse med anerkendte internationale (modificerede) testguidelines, der er udviklet til opløselige kemikalier, er nanomaterialernes iboende egenskaber ofte årsag til varierende eksponeringsforhold under selve den økotoksikologiske test, hvilket kan føre til misvisende testresultater.

Denne rapports konkrete anbefaling er således, at PNEC-bestemmelser for ENMs udføres efter at effektstudierne er evalueret for deres risikovurderingsegnethed i henhold til den transparente og reproducerbare metode som er udviklet, beskrevet og anvendt i denne rapport.

1. Introduction

1.1 Background – Environmental Risk Assessment and PNEC values for nanomaterials

Engineered nanomaterials (ENMs) may be introduced into the environment both intentionally and non-intentionally throughout the life-cycle of ENM production, use and disposal. As the use of nanomaterials increases across a wide range of sectors, environmental release is an inevitable consequence. Today urban stormwater, landfill leachates, effluents from wastewater treatment plants and waste incineration are all anthropogenic processes likely to contain ENMs from nano-enabled consumer and industrial products either disposed of at the end of their use phase, released through accidental spills during production or later during transport of ENMs. Wear and tear of materials containing ENMs may also lead to potential release during the use-phase. In addition, intentional release into the environment must be considered, such as via the use of e.g. nano-zero valent iron (nZVI) in the remediation of groundwater polluted with chemicals such as chlorinated solvents (Grieger, Baun et al. 2010).

With respect to environmental effects of ENMs, concerns are related to the fact that nano-scale dimensions of materials tend to be more reactive and may behave differently than their bulk-scale counterparts (Royal Commission 2008). For example, compared to bulk materials ENMs may exhibit different catalytic potential, solubility, electrical conductivity, material strength, and magnetic behaviour (Oberdorster, Oberdorster et al. 2005). By definition most ENMs are indeed considered to be 'novel' materials, many of which were developed to take advantage of their highly reactive properties, and novel effect characteristics may therefore be expected for ENMs.

The increasing use and hence anticipated increased release of ENMs from consumer products to the environment makes environmental safety assessment of utmost importance. In the European Union the procedure for this type of assessment entails an exposure assessment for deriving the Predicted Environmental Concentration (PEC) and a dose-response (effect) assessment leading to Predicted No-Effect Concentrations (PNECs). The latter is based on a critical evaluation of existing ecotoxicological data and the procedures for PNEC estimation is described in detail in (ECHA 2008). These procedures have been developed for "regular" chemicals, but are also recommended to be used for ENMs since they in principle should be applicable (Aitken, Bassan et al. 2011). However, it should be emphasized that by using existing procedures and guidelines for effect assessment, engineered nanoparticles are considered to be one of several different forms of the parent materials and that present technical guidance for completing a risk assessment can be used with some minor technical and/procedural amendments only (Grieger, Baun et al. 2010).

The OECD (Organisation for Economic Cooperation and Development) is a key player in the establishment of test guidelines and guidance for safety testing of chemicals and is also heavily involved in test method development for nanomaterials. The challenges for risk assessment posed by ENMs have to some extent been addressed by the program of work at the OECD's working party on manufactured nanomaterials (OECD 2010). An array of projects are being directed, including the development of a nanomaterial risk research database analysis of test methods (which underpin chemical risk assessment) as applied to nanomaterial and a sponsorship program that will acquire risk assessment data for nanomaterials of current relevance. In an OECD expert meeting held in Berlin in 2013 it was concluded that several of the current test guidelines **do not** apply to

nanomaterials and many others need to be adapted to be applicable (OECD 2014). Work is currently ongoing to develop and adapt these documents.

In environmental risk assessment, reliable (and reproducible) results of ecotoxicological experiments are mandatory prerequisites. Besides the above-mentioned challenges, the field of nano-ecotoxicology is further challenged by the fact that before 2004 no ENMs had been studied (Stone, Hankin et al. 2010). As outlined by Hartmann, Von der Kammer et al. (2010) problems of reproducibility occur in standardized test systems when particle suspensions are tested and not substances in solution as it is the case for chemicals for which the tests were developed. Problems of uncontrollable aggregation affected by dilution, media composition and the organisms themselves are frequently reported (Hartmann, Von der Kammer et al. 2010, Hartmann, Engelbrekt et al. 2013, Tiede, Hassellov et al. 2009, Hasselloev, Readman et al. 2008, Baun, Hartmann et al. 2008) and this hampers the interpretation of effects observed. While the number of ecotoxicity studies are indeed rapidly increasing in these years it is not given that these studies are relevant for the effect assessment carried out as a part of the environmental risk assessment of ENMs (Wickson, Hartmann et al. 2014).

Environmental risk assessment is required to ensure safety of nanomaterials and to protect the environment from unintentional adverse effects. In a regulatory context this requires reliable and relevant environmental hazard data upon which PNEC values can be estimated. For nanomaterials it is well-known that ecotoxicity testing is not straight-forward and that the applicability of commonly used test guidelines and guidance can be questioned (e.g., Baun, Hartmann et al. (2009). Nanomaterials are known to behave very differently in ecotoxicity test systems compared to soluble chemicals, for which most guidelines were intended.

This current lack of appropriate guidance implies that previous and current guideline-based hazard testing may not be appropriate for testing of ENMs. It further entails that the data, upon which currently available PNEC values have been established, may not correctly reflect the actual ecotoxicity of these ENMs. This means that existing data from non-standard tests – or tests following modified test guidelines –in some cases may provide information of equal or higher reliability compared to strictly guideline-based tests. This would be the case if these modifications were applied to cater for nanomaterial properties and behaviour in the test system. Such data should therefore not *per se* be considered less reliable as basis for PNEC estimation.

1.2 Objective and scope

The overall objective of this report is to provide data on environmental effects of nine selected ENMs in order to derive their respective PNEC values. This will eventually feed into an environmental risk evaluation of the selected ENMs, which constitutes the last component of the NanoDEN project.

In the context of this report ENMs are defined as manufactured materials with one or more external dimensions between 1 and 100 nm. In that respect an ENM is a part of a broader group of nanomaterials, which can result from natural, anthropogenic (incidental) and engineered (intentional) processes, however the NanoDEN project focuses on ENMs, as this is the most relevant group of nanomaterials from an up-stream regulatory point of view. The following materials have been selected for this project:

- Titanium Dioxide (TiO_2) (rutile and anatase)
- Zinc Oxide (ZnO)
- Silver (Ag)
- Carbon Nanotubes (CNTs)
- Copper Oxide (CuO)

- Nano Zero Valent Iron (nZVI)
- Cerium Oxide (CeO_2)
- Quantum Dots (QDs)
- Carbon Black (CB)

The selection was based on expected production and use volumes in Denmark and their applications in relevant consumer products, industrial processes, and environmental remediation processes. In the NanoDEN project $CuCO_3$ nanoparticles are included as a case material for copper containing nanoparticles. However, in this report $CuCO_3$ has been replaced by CuO nanoparticles due to the fact that CuO nanoparticles have been studied to some extent in ecotoxicity studies, whereas studies for $CuCO_3$ are lacking.

Through a critical review this report will summarize the current knowledge with respect to PNEC estimation methodologies, data selection criteria and their applicability to ENMs. This included a specific review of nano-PNEC values in the scientific literature. Knowledge gaps in relation to methodological limitations, data availability, relevance and read-across will be identified. Alternative methodologies for PNEC estimation will be identified and compared to current procedures, with a specific focus on their applicability to the special concerns that ENMs raise. A transparent approach to assess the adequacy of effect data for PNEC estimation will likewise be developed. This will result in recommendations and suggestions for pragmatic approaches to estimating PNEC values for ENMs while at the same time highlighting current uncertainties and future research needs. Finally, on this basis, estimated PNEC values will be included for the selected ENMs chosen for this project.

In this report a strong focus is directed towards effects in the aquatic environment. This is because the aquatic phase is seen as the starting point for understanding the environmental fate and behaviour of ENMs as a main point of potential entry into the environment, creating a link between the other environmental compartments such as soil, sediment and air (Hartmann, Skjolding et al. 2014). Furthermore, the behaviour of ENMs in complex matrices like soils and sediments is not well-understood at present. The far majority of effect studies are performed on freshwater aquatic organisms, also because the base-set test organisms belong to the freshwater compartment. However, given similar sensitivities among organisms living in different compartments, there is an option of extrapolating from one compartment to another, as e.g. known for organic chemicals from freshwater to soil/sediment organisms using distribution coefficients. But this requires that relevant physico-chemical parameters are available and that given relations exist.

1.3 Report structure

Following the above introduction the report is divided in 6 chapters.

For those readers not familiar with how PNEC values are derived according to the European chemicals regulation, Chapter 2 gives an overview of the accepted approaches for PNEC derivation as well as how effect studies are evaluated for their adequacy for risk assessment.

Chapter 3 contains an overview of existing PNEC values for the ENMs selected for study in this report – both from REACH registrations as well as from the open literature. For the sake of comparison PNEC values for the ionic and bulk forms of the ENMs are shown when applicable.

As the current way of evaluating the risk assessment adequacy of effect studies is not suited for ENMs, Chapter 4 presents a state-of-the-art evaluation procedure for effect studies of ENMs with respect to their risk assessment adequacy.

In Chapter 5 effects studies for the selected ENMs are evaluated for their risk assessment adequacy and the most appropriate study is selected for the PNEC derivation.
Chapter 6 sums up the report with a comparison of the PNEC values obtained in this report with PNEC values found in the literature and from REACH (see Chapter 3). This is followed by a discussion on how different properties of the ENM, experimental, biological as well as risk assessment issues influence the PNEC derivation and how these identified GAPs have implications for chemical safety assessment.

Finally, Chapter 7 presents the conclusions of the report.

2. Current approaches for estimation of PNEC values and data evaluation for traditional chemicals

For readers who are not familiar with environmental risk assessment this section provides a brief introduction to the key concepts for determining the PNEC value and currently recommended approaches for PNEC estimation under the European chemicals regulation (REACH). Further the section introduces the method used for evaluation of the regulatory adequacy of data from ecotoxicological tests.

2.1 Predicted No-Effect Concentration in the risk assessment paradigm

In the European chemicals regulation (REACH) the chemical safety assessment (CSA) is the main instrument to identify and control the risk of chemicals (ECHA 2008). The CSA is divided in three steps: Hazard assessment, exposure assessment and risk characterisation. In this framework the environmental hazard assessment aims to identify the hazards of the chemical substance to ecosystem functions and organisms in the environment. This assessment encompasses a dose-response evaluation, which is based on collection of ecotoxicological tests results that upon evaluation are used to establish the threshold concentration below which exposure is considered to be safe. This concentration is referred to as the Predicted No-Effect Concentration (PNEC) which by ECHA is defined as "…the concentration of a substance in any environment below which adverse effects will most likely not occur during long term or short term exposure" (ECHA 2008). It is a requirement in REACH that the PNEC value is derived for substances manufactured, imported, or used in more than 10 t/year. In order to derive a PNEC for a substance all available hazard information must be evaluated. This will in most cases comprise laboratory studies following both standard and non-standard methods. It is a general assumption that ecosystems, i.e. naturally occurring organisms, are more sensitive to chemical substances than organisms used in the laboratory. Furthermore, most laboratory studies are carried out on single organisms. In order to compensate for, among other things the difference in sensitivity between organisms in their natural habitats and organisms bred and kept in the laboratory, assessment factors are applied to the laboratory test results. It is therefore not directly the test from the laboratory that is used, but an extrapolation of the laboratory test result is carried out.

Two approaches for determining PNEC values are described within REACH and the EU technical guidance documents, namely: 1) the assessment factor (AF) approach and 2) the deterministic approach using species sensitivity distributions (SSDs) (ECHA 2008). The AF approach can in principle be used when only short-term effect data exist for the three trophic levels defined as the base-set organisms, i.e. algae, daphnia and fish, and the most sensitive organism is used for the calculations of the PNEC value. In contrast to the simple AF approach the SSD approach requires at least ten high quality NOECs/EC_{10}-values from different organisms belonging to eight taxonomic

groups. Hence, in the recognition of the general lack of such many and varied data for traditional chemicals, it is often the AF approach that is used in practice, despite the wish to utilise a broader range of data better representing the variety of organisms.

For both approaches, uncertainties in the extrapolation are assumed to be covered by applying AFs according to (ECHA 2008). These uncertainties comprise:

- Intra- and inter-laboratory variation of toxicity data;
- Intra- and inter-organism variation (biological variation);
- Short-term to long-term extrapolation;
- Laboratory data to field impact extrapolation (additive, synergistic and antagonistic effects from the presence of other substances may also play a role here)

Obviously, the AF depends on the type of the effect data; with short-term and few data a high AF is required, while lower AFs can be used if long-term and many data exist. While the scientific basis for the use of AF is debateable, a consensus has developed among different regulatory frameworks with regards to the magnitudes of AFs (van Leeuwen, Vermeire 2007).

2.2 The Assessment Factor approach

The general principle of the AF approach is that the result from a laboratory test is divided by an AF. The less data available, the higher the AF applied. PNECs are then estimated by dividing the lowest value found in toxicity tests with the chosen AF. The available effect data are simply evaluated against short-term/long-term and trophic level criteria. The aim is to represent the ecosystem by data from one organism at each of three different trophic levels: algae, crustaceans and fish; i.e. the base-set test organisms. Additionally, these data should preferably be NOECs from chronic studies. Alternatively EC_{10}-values or extrapolations from LOECs can be used instead of NOECs. If only short-term values exist, the lowest EC_{50}-value should be used.

The PNEC is thus derived according to Equation 2-1.

$$PNEC = \frac{Lowest\ NOEC\ or\ EC_{50}}{AF}$$ Equation 2-1

The AFs recommended by (ECHA 2008) for estimation of PNEC values for the (freshwater) aquatic environment are shown in Table 2.

Table 2 - Assessment factors for use in PNEC estimation in freshwater under the assessment factor approach (ECHA 2008). For details on footnotes to the individual AFs, see Appendix 2.

Available data	Assessment factor
At least one short-term L(E)C50 from each of three trophic levels (fish, invertebrates (preferred Daphnia) and algae)	1000 [a)]
One long-term EC10 or NOEC (either fish or Daphnia)	100 [b)]
Two long-term results (e.g. EC10 or NOECs) from species representing two trophic levels (fish and/or Daphnia and/or algae)	50 [c)]
Long-term results (e.g. EC10 or NOECs) from at least three species (normally fish, Daphnia and algae) representing three trophic levels	10 [d)]
Species sensitivity distribution (SSD) method	5-1 (to be fully justified case by case) [e)]
Field data or model ecosystems	Reviewed on a case by case basis [f)]

If long-term data are available for all organisms in the base-set the lowest NOEC should be divided by an AF of 10 to obtain the corresponding PNEC. However, if the data only comprise chronic NOEC values for two trophic levels, the lowest NOEC should be divided by an AF of 50. If the data only consist of either a chronic NOEC from a fish or daphnia study, it should be divided by an AF of 100. Finally, if no long-term data exist, but only short-term values for the three base-set trophic levels exist, an AF of 1,000 is applied.

2.3 The Species Sensitivity Distribution approach

The Species Sensitivity Distribution (SSD) is based on the assumption, that the sensitivities of the organisms in the ecosystem follow a theoretical distribution and that the organisms tested in the laboratory is a random sample of this distribution. In order to establish a SSD for a given substance a substantial amount of effect data is needed. All of these effect data must be high-quality NOECs/EC_{10}-values from chronic or long-term studies.

According to (ECHA 2008) data have to be compiled for at least ten, but preferably 15, ecotoxicological studies which should comprise eight different taxonomic groups, covering algae, crustaceans, fish, insects, higher plants as well as other families of insects and fish.

In comparison to the base-set of ecotoxicological tests used for the AF approach, there may not exist standardized methods for these other taxonomic groups. Thus, the requirements for regulatory relevance and reliability of test results have to be assessed on a case-by-case basis.

If there is a sufficient amount of validated effect data for a given substance, the range of data is fitted to the selected theoretical distribution. It should be noted that different distribution models are recommended by different regulatory authorities; where the US EPA suggests a log-triangular function, ECHA guidance document for safety assessment of chemicals in the EU operates with a log-logistic or a log-normal function. If the fitting of the data to the selected model results in a lack-of-fit, the SSD should not be used. However, if a good fit is obtained the so-called hazardous concentration to 5% of the organisms (HC_5) or the 5th percentile of the chronic toxicity data distribution can be derived, which is then considered protective for most organisms in a community. The HC_5 can be derived with a 50 or 95% confidence interval, using Equation 2-2.

$$HC_5 = 10^{\left[x_m - k_m \cdot s_m\right]} \qquad \text{Equation 2-2}$$

where m is the number of organisms, x_m is the sample mean of log NOEC data for m species, k_m is the one-sided extrapolation constant for a logistic or normal distribution and s_m is the sample standard deviation of log NOEC values for m organisms.

When the HC_5 has been estimated, the PNEC is calculated according to Equation 2-3, where the AF is a number between 1 and 5.

$$\mathrm{PNEC} = \frac{\mathrm{HC}_5}{\mathrm{AF}} \qquad \text{Equation 2-3}$$

As a standard an AF of 5 is used. This value can be reduced towards 1, however, it requires full justification of the reduced uncertainties if an AF lower than 5 is to be used.

2.4 Data selection for PNEC estimation – the Klimisch score

In the currently recommended approach for safety assessment of chemicals the regulatory relevance and reliability of a study needs to be evaluated before it can be used in either of the two approaches for PNEC estimation described above. In the ECHA guidance document (ECHA 2011) it is recommended that the quality of a given effect study is evaluated by assigning a so-called Klimisch score to the study. Usually only studies assessed as K1 or K2 will be used for further PNEC estimation. The Klimisch scores are described in the following (from Klimisch, Andreae et al. (1997) as cited by ECHA (2011), "Guidance on information requirements and CSA. Chapter R.4: Evaluation of available information"):

K1 = reliable without restrictions: "studies or data [...] generated according to generally valid and/or internationally accepted testing guidelines (preferably performed according to GLP) or in which the test parameters documented are based on a specific (national) testing guideline [...] or in which all parameters described are closely related/comparable to a guideline method."

K2 = reliable with restrictions: "studies or data [...] (mostly not performed according to GLP), in which the test parameters documented do not totally comply with the specific testing guideline, but are sufficient to accept the data or in which investigations are described which cannot be subsumed under a testing guideline, but which are nevertheless well documented and scientifically acceptable."

K3 = not reliable: "studies or data [...] in which there were interferences between the measuring system and the test substance or in which organisms/test systems were used which are not relevant in relation to the exposure (e.g. unphysiological pathways of application) or which were carried out or generated according to a method which is not acceptable, the documentation of which is not sufficient for assessment and which is not convincing for an expert judgement."

K4 = not assignable: "studies or data [...] which do not give sufficient experimental details and which are only listed in short abstracts or secondary literature (books, reviews, etc.)."

Currently, ECHA uses the Klimisch categorisation for all effect studies and only studies obtaining K1 or K2 will be considered in the overall risk assessment. The evaluation of reliability and relevance of ecotoxicity data for regulatory environmental risk assessment is, as described in the previous section, often based on the assignment of a Klimisch score. This means that toxicity data from standard guideline tests are preferred over non-standard test data. In the case of ENMs current test methods may not be directly applicable to testing of their potential hazardous properties. It has therefore also been suggested that other endpoints and/or test organisms may be more relevant (Ågerstrand, Breitholtz et al. 2011). In this light, data generated by non-standard tests could improve the scientific basis of risk assessment by providing relevant and more sensitive endpoints. At the same time it is acknowledged that some criteria for data quality must be agreed upon to ensure comparability and mutual acceptance of data (MAD).

3. Currently existing PNEC values for nanomaterials – literature values and REACH registrations

In this section established PNEC values are presented based on scientific literature and data from registration dossiers submitted to ECHA. PNEC values for the ionic form (when applicable), the bulk form (when applicable) as well as PNEC values for the nano form will be presented. These values will later be compared to the PNEC values established based on the methodology developed as part of this project with the purpose of discussing differences resulting from data selection and estimation method. However, first an overview of existing and alternative approaches to PNEC estimation for ENMs will be presented.

3.1 PNEC Estimation Methodologies

As mentioned in Chapter 2, there are two approaches for determining PNEC values described in REACH, and these also constitute the basis for PNEC estimation of ENMs.

With regard to the AF approach, the RIPoN3 report (Aitken, Bassan et al. 2011) found that in principle there does not seem to be nano-specific arguments to change *the way* assessment factors are selected in the AF approach today, i.e. that more available data from long-term tests can reduce the AF from 1,000 to 100, 50, and 10. However, the AFs were originally intended not only to cover the uncertainty related to the amount of available data but also factors such as inter- and intra-organism differences and extrapolations from laboratory to field, but when deciding on *the magnitude* of the current AFs it was not considered also to cover potentially nano-specific effects or test artefacts arising from the distinct different nature of ENMs compared with soluble chemicals.

The values of the AFs are based on regulatory practice and empirical knowledge on ecotoxicological effects of chemicals. Since there is no history for evaluation of ENMs, it is at present not possible to claim that the use of the presently available AFs will ensure that organisms will be protected at concentrations below PNEC. The RIPoN3 report also found the SSD approach to be principally acceptable for deriving PNEC values for ENMs. It should be noted that this approach requires at least ten high quality NOECs/EC_{10}-values from different organisms belonging to eight taxonomic groups. This kind of data is not available for any ENM today and thus it remains to be shown that the deterministic approach for PNEC determination will actually be applicable to ENMs.

Independent of the PNEC estimation methodology, i.e. application of AFs to derive safe levels, the reliability of the underlying NOEC values is crucial. The fact that ENMs agglomerate/aggregate when dispersed in solution potentially results in the transfer from small reactive particles to larger non-reactive particles. In such a case, particles nominally expected to be small will show a reduced effect, e.g. no effect, but when an AF is applied in order to derive the PNEC value, the derived PNEC

may potentially represent a concentration that in fact has an effect on the environment, i.e. at a particle concentration level where the degree of agglomeration is not the same as in lower concentrations. Typically most agglomeration is seen at higher concentrations. The lower agglomeration at low concentration will leave the ENM in the nano-sized range where the potentially more reactive particles may exert biological effects not observed for the larger particles/agglomerates. This phenomenon has much to do with the current difficulties in verifying the exposure level and will as such have a large influence on the PNEC and should therefore have an implication on the choice of the magnitude of the AF, which is not accounted for within the present approach.

The currently available AFs are intended to cover uncertainties, e.g. from lab to field. As suggested in the literature, see later, an extra nano-specific AF could be applied to the current approach in order to cover estimation from e.g. ion to nano.

3.2 Alternative approaches to PNEC estimation for ENMs

Given the concerns on data availability, data quality and relevance of methods for ENMs, a number of alternative approaches have been suggested for the estimation of nano-PNEC values. These include:

- The **Probability Species Sensitivity Distribution (PSSD)** approach as described by Gottschalk, Kost et al. (2013). It is stated that "this method accounts for inter- and intra-organism differences in toxic effects that may be observed for different modifications of an ENM to the same environmental organism." To transform the raw data on ecotoxicity into NOEC values, to be used for the PSSD, AFs were applied:
    - A factor of 10 to reflect the uncertainty of short- to long-term effect extrapolation.
    - A factor of 10 to derive NOEC values from L(E)C_{50} values
    - A factor of 2 to derive NOEC values from L(E)C_{10-20} values.

  The long-term NOEC values were then used to create SSDs for each individual organism which are subsequently combined into a "generic probabilistic species sensitivity distribution" for the specific ENM and environmental compartment (Gottschalk, Kost et al. 2013). This method does not directly result in a PNEC value but rather a PSSD from which conclusions can be made about e.g. percentile values, i.e. concentrations for which a certain % of the organisms will be affected. This approach has further been developed in order to derive PSSD-based PNEC values. The PSSD can also be compared to a probability distribution for environmental concentrations to identify 'critical concentration ranges'.

  The appropriateness of this method to nano-PNEC estimations can be questioned by the fact that the current uncertainty 'embedded' in the raw ecotoxicity data is likely to highly influence the resulting PSSD. Though this is also the case with the AF and SSD approaches, the PSSD approach include all available data, which severely may influence the derived PNEC. A benefit of the PSSD approach is the possibility of including a wider dataset (i.e. all available EC_X and NOEC values), however, this is at the same time a drawback, as AFs have to be applied in order to obtain NOEC values from the EC_X data, i.e. using estimated data for further estimations.

- Using PNEC values for the corresponding **dissolved metal ion** as a starting point and applying a safety factor (Notter, Mitrano et al. 2014). This approach is relevant only to soluble metal/metaloxide ENMs. The approach is based on a systematic comparison of ecotoxicity data for metal or metal oxide ENMs and the corresponding soluble metal ion toxicity. It was evaluated how often the metal ion was more toxic than the nanomaterial

and vice versa. On this basis Notter, Mitrano et al. (2014) proposed to divide the corresponding metal PNEC with a factor 2 to ensure a conservative PNEC for the ENM.

- Deriving so-called **INECs (Indicative No Effect Values)** (Aschberger, Micheletti et al. 2011). The term 'indicative' is used to indicate the uncertainty that is associated with the estimated NEC value and that it should not be applied in regulatory risk assessment. It is however compared to PEC values to compare the orders of magnitude between potential effect concentrations and environmental concentrations.

As mentioned before, it is recognised that current standard methods and guidelines for ecotoxicity testing are often not appropriate for testing of ENMs. Test methods have been developed and refined over the last decade of nanoecotoxicology research and work is ongoing within the OECD WPMN. However consensus on most appropriate methods has not yet been reached. This implies that using all available data means using data that varies in reliability.

3.3 Predicted No Effect Concentrations in REACH registrations

The following sections will present a brief overview of PNEC values published in REACH registrations based on searches in the ECHA database of registered substances (http://echa.europa.eu/web/guest/information-on-chemicals/registered-substances). A chemical substance may exist in several forms and in the context of ENMs the terms "ionic form", "bulk form" and "nano form" are often used to distinguish between these forms – especially for metal and metal oxide materials. The terms are explained below in Box 1. Searches were carried out in the ECHA database both for the particulate forms and the corresponding ionic forms. For materials that can exist both as bulk and nano forms it was checked if the database dossiers intended to cover both forms (i.e. the given PNEC is intended to cover both forms) or if specific PNEC values were provided for the two forms separately. Some materials do not by definition exist in a bulk form (e.g CNTs) and these database entries are therefore implicitly for the nano form of the material.

Box 1 – Explanation of terms used to describe different forms of the same chemical substance.

Ionic form: This refers to dissolvable metal salts or dissolved metal ions/metal ion complexes. The metal corresponds to the metal that is/forms part of the corresponding particulate material for which the ionic form is used as a reference compound and/or for read-across purposes. Examples of ionic forms are $CuSO_4$ or Cu^{2+} (and dissolved Cu-complexes), which can be used as a reference substance in studies with e.g. Cu(*s*) or CuO(*s*) particles. Ionic forms are only relevant for metal-containing substances.

Bulk form: This refers to a particulate form of a material, where the particle sizes are larger than what is defined as nanoparticles (typically >100 nm). Some materials exist in different particle sizes. An example is TiO_2 that can be produced both as nanoparticles (i.e. in the nano form, see below) and as larger sized particles (i.e., bulk form). It should be noted that some materials by definition only exist as nanomaterials and a corresponding bulk form does therefore not exist. This is for example the case with CNTs.

Nano form: This refers to a particulate form of a material with particle sizes/size distributions that classifies them as nanoparticles (may differ between different definitions but typically <100 nm). In the context of this report nanoparticles are defined by their size with one or more external dimensions between 1 and 100 nm.

3.3.1 PNECs for the ionic form

The following ions are considered relevant with regard to the ENMs selected as case study materials in this project: Zn^{2+}, Ag^{+}, Cu^{2+}. These ions correspond to the case study materials ZnO, Ag and CuO ENMs and were chosen based on the evaluation of relevant processes for the selected case study materials as presented in the NanoDEN SP1 report (Hartmann, Skjolding et al. 2014). Based on searches in the ECHA database of registered substances (http://echa.europa.eu/web/guest/information-on-chemicals/registered-substances) PNEC values for metal salts of these ions have been identified as listed in Table 3 below.

Table 3 - Overview of PNEC estimations for ion metals (metal salts) from registration dossiers submitted to ECHA.

Test substance	$PNEC_{freshwater}$, µg/L	$PNEC_{marine\ water}$, µg/L	$PNEC_{STP}$, µg/L	$PNEC_{sediment\ freshwater}$, mg/kg dw	$PNEC_{sediment\ marine}$, mg/kg dw	$PNEC_{soil}$, mg/kg dw
$ZnCl_2$	20.6 *(SE; Af=1)*	6.1 *(SE; Af=1)*	100 *(AF; Af=1)*	118 *(SE; Af=1)*	56.5 *(PC; Af=1)*	35.6 *(SE; Af=1)*
$AgCl_2$/ $AgNO_3$	0.04 *(SE; Af=3)*	0.86 *(AF; Af=10)*	25 *(AF; Af=1)*	438 *(AF; Af=1)*	438 *(AF; Af=1)*	0.794 *(PC)*
$CuSO_4$/ CuCl	7.8 *(SE; Af=1)*	5.2 *(AF; Af=1)*	230 *(SE; Af=1)*	87 *(Af=1)*	676 *(PC; Af=1)*	65 *(Af=1)*

The abbreviations SE, AF and PC refer to statistical extrapolation, the assessment factor and the partition coefficient approaches, respectively; The Af values are the applied AFs (e.g. Af=10).

3.3.2 PNECs for the bulk form

The following bulk forms are considered relevant with regard to the ENMs selected as case study materials in this project: TiO_2, ZnO, Ag, CuO and CeO_2,. Based on searches in the ECHA database of registered substances PNEC values for bulk forms of these materials have been identified as listed in Table 4 below.

Table 4 - Overview of PNEC estimations for bulk materials from registration dossiers submitted to ECHA.

Test material	$PNEC_{freshwater}$, µg/L	$PNEC_{marine\ water}$, µg/L	$PNEC_{STP}$, µg/L	$PNEC_{sediment\ freshwater}$, mg/kg dw	$PNEC_{sediment\ marine}$, mg/kg dw	$PNEC_{soil}$, mg/kg dw
TiO_2*	127 *(AF, Af=100)*	1.000 *(AF, Af=10,000)*	100.000 *(AF, Af=10)*	1,000 *(AF, Af=100)*	100 *(AF, Af=1,000)*	100 *(AF, Af=10)*
ZnO*	20.6 *(SE; Af=1)*	6.1 *(SE; Af=1)*	100 *(AF; Af=1)*	118 *(SE; Af=1)*	56.5 *(PC; Af=1)*	35.6 *(SE; Af=1)*
Ag*	0.04 *(SE; Af=3)*	0.86 *(AF; Af=10)*	25 *(AF; Af=1)*	438 *(AF; Af=10)*	438 *(AF; Af=10)*	1.41 *(SE; Af=3)*
CuO	7.8 *(SE; Af=1)*	5.2 *(AF; Af=1)*	230 *(SE; Af=1)*	87 *(Af=1)*	676 *(PC; Af=1)*	65 *(Af=1)*
CeO_2*	-	-	-	N/A	N/A	-

*: dossier covering bulk and nano form(s) of the substance (see also section 3.3.3 for further details); -: not addressed in the dossier; N/A: No or insufficient data available at present; The abbreviations SE, AF and PC refer to statistical extrapolation, the assessment factor and the partition coefficient approaches, respectively; The Af values are the applied AFs (e.g. Af=10).

It should be noted that PNEC values in these registration dossiers, often covering both bulk and nano forms of the materials, are almost identical to the PNEC values for the corresponding metal salts as listed in Table 3. In the publically available version of the ECHA registration database it is not possible to access information which explains the rationale behind using the same PNEC values for several compounds containing the same metal (as for e.g. $CuSO_4$, CuCl and CuO). However, it is

likely to be based on dissolved concentrations of the metal ion (e.g. Cu^{2+}), which is considered to be responsible for the ecotoxic effects.

3.3.3 PNECs for the nano form

The final report of the NanoSupport project, published by the European Commission in 2012 (EC 2012), presents a review of REACH registration dossiers which are known or considered to cover nanomaterials or nano forms of a material. Regarding PNEC values it was found that no values were derived based on data from tests on the nanomaterial or the nano form of the substance. In many cases PNEC values were established based on read-across e.g. to a soluble metal salt. In those dossiers, which covered both a nano and a non-nano (bulk) form of a material, the PNEC value was based on data for the non-nano form. This implies that the PNEC values in Table 4 above are likely to be based on tests with bulk materials. Therefore no nano-specific PNEC values were identified from the ECHA registration database for the materials: TiO_2, ZnO, Ag, CuO and CeO_2. Instead the PNEC values in Table 4 for the bulk form are considered by the registrants also to apply to the nano form. However, the following materials are considered to be nanomaterials by definition: CNT, nZVI and CB. The ECHA database was searched for information on PNEC values for these materials, see Table 5. No dossier could be found for nZVI. For CNT two dossiers for multi walled CNTs (MWCNTs) were found whereas for CB one joint dossier and two individual submissions were identified. For QDs it is not possible to find any information in the database. This search is further complicated by the fact that QDs can be produced in many different material combinations. This would make their properties (and PNEC values) composition-specific.

Table 5 - Overview of PNEC estimations for nanomaterials from registration dossiers submitted to ECHA.

Test material	$PNEC_{freshwater}$, mg/L	$PNEC_{marine\ water}$, mg/L	$PNEC_{STP}$, mg/L	$PNEC_{sediment\ freshwater}$, mg/kg dw	$PNEC_{sediment\ marine}$, mg/kg dw	$PNEC_{soil}$, mg/kg dw
MWCNT *(JS)*	0.43 *(AF; Af =10)*	0.043 *(AF; Af =100)*	100 *(AF; Af =100)*	N/A	N/A	N.E.
Graphite / MWCNT *(IS)*	0.78 *(AF; Af =10)*	0.078 *(AF; Af =100)*	50 *(AF; Af =10)*	N/A	N/A	N/A
CB *(JS)*	5 *(AF; Af =1,000)*	5 *(AF; Af =10)*	-	-	-	-
CB *(IS)*	N.D.	N.D.	N.D.	N.E.	N.E.	N/A
CB *(IS)*	50 *(AF; Af =100)*	N.D.	N.D.	N/A	N/A	N/A

JS: Joint submission; IS: Individual submission; N/A: No or insufficient data available at present; N.D.: No data: aquatic toxicity unlikely; N.E.: No exposure expected; The abbreviations SE, AF and PC refer to statistical extrapolation, the assessment factor and the partition coefficient approaches, respectively; The Af values are the applied AFs (e.g. Af=10).

3.4 PNEC established in the scientific literature for ENMs

The scientific literature was particularly reviewed with focus on PNEC values for the ENMs chosen as case materials for this report. As the main focus of this report is freshwater aquatic PNEC values (see chapter 5) this has also been the focus of the search for PNEC values in the literature.

A search performed in ISI Web of Knowledge using the search terms 'PNEC' AND 'nano*' gave 11 hits of which five were found to be relevant based on the abstract information. However, it turned out that only three of the articles contained PNEC estimations whereas the other two were for non-nano compounds and a conference abstract, respectively. An additional, although not systematic, search in Google was performed to identify reports and other literature not included in the ISI Web of knowledge database. Also, other known data sources were included such as the ENHRES project report from 2010 (Stone, Hankin et al. 2010) and an additional scientific article (Gottschalk, Kost et al. 2013). Although this search strategy may not cover all existing literature that has established PNEC values for the case study ENMs, the main purpose of this exercise was to give an overview of PNEC ranges and approaches used to establish such values. At the same time discussions of limitations to these approaches were identified. The ENMs were characterised to various extents depending on the state of knowledge at the time being, purpose of the article, journal and authors. However, the focus of this exercise was to establish an overview of how many PNEC values have been published for the selected case ENMs and at which levels. This overview of PNEC values, approaches and gaps can be seen from Table 6, where the size of the data set laying behind each PNEC is also indicated.

Table 6 – Freshwater (aquatic) PNEC estimations for ENMs in the scientific literature (articles, reports etc.). The approaches to PNEC estimation is described as well as the specific ENMs for which the values have been estimated. When specific gaps, which have hampered the PNEC estimation, have been highlighted in the literature these are also described.

ENM	PNEC value	Approach	Highlighted gaps	Reference
TiO_2 Ag	1 µg/L 1 µg/L	REACH TGD approach based on "published ecotoxicological data. Four (including one NOEC) and nine effect data, respectively resulting in an assessment factor (AF) of 1,000 was applied to account for the large data uncertainties".	Limited studies at different trophic levels. Large data uncertainties. Lack of standardized and validated chronic toxicity data.	(Musee 2011)
TiO_2 Ag CNT	1 µg/L 0.696 ng/L 40 µg/L	PNEC was established from EC or NOEC values by dividing by an assessment factor of 1,000 due to the low number of available studies. The exact number of studies are not specified but described as limited.	Risk assessment for ENMs based on a PEC/PNEC risk quotient suffers from PNEC estimation uncertainties. Derivation of PNECs is difficult due to: - Limited number of studies - Studies focused on acute toxicity, few test organisms and toxicity endpoints, model organisms and using high ENM concentrations???? - Long-term low-exposure studies to obtain chronic endpoints are missing - Unclear to what extend the use of a safety factor of 1,000 covers all limitations	(Gottschalk, Sonderer et al. 2010)

ENM	PNEC value	Approach	Highlighted gaps	Reference
			Also it is difficult to compare a deterministic PNEC to PEC values from density distributions.	
CeO_2 (14 nm) CeO_2 (20 nm) CeO_2 (29 nm)	0.052 mg/L 0.068 mg/L 0.108 mg/L	Data generated in this reference on 3 trophic levels. One short-term effect data on fish and two long-term EC_{10} values from algae and daphnia are available. Hence, an extrapolation factor of 50 is applied to the lowest EC_{10} values according to ECHA technical guidance.	"in this study standard test conditions were applied, despite the recent concerns raised about the relevance of these methods for assessing the risks of nanoparticles". "It has been suggested that test organisms should be exposed to nanoparticles in an environmentally relevant way in order for the PEC and the PNEC to be based on the same nanoparticulate form". "No data is available on the appropriateness of the extrapolation factors used for calculating PNECs".	(Van Hoecke, Quik et al. 2009)
TiO_2 ZnO Ag CNT CuO nZVI CeO_2	61 µg/L 9.9 µg/L 0.01 µg/L 60 µg/L 0.48 µg/L 8.3 µg/L 2 µg/L	Modelling of probabilistic species sensitivity distributions (PSSD). The values are HC_5 percentile values based on the PSSD. A factor of 10 was applied for short to long-term extrapolation as well as for EC_{50} to NOEC transformation. A factor of 2 was applied for EC_{10}/EC_{20} to NOEC transformation. To generate the PSSDs 23, 29, 25, 10, 21, 10 and 18 effect studies were used respectively.	The species sensitivity distributions do not reflect validated results. "species sensitivity distribution results that stem from integrating the different material properties and folding them into one model inevitably come with large variabilities and uncertainties. Such insecurities could be reduced if sufficient data were available by modelling probability distributions for individual material properties". "our understanding of the crucial parameters that define the toxicity of ENMs is not yet sufficiently well developed to allow for such a more detailed formulation of species sensitivity distribution". "Another main source of variability or uncertainty in the species sensitivity distribution model output is that different test conditions and the combination of different materials with varying testing conditions may produce highly varying effects for one ENM on one organism."	(Gottschalk, Sonderer et al. 2010) (Jacobs, Gottschalk et al. In prep)
TiO_2 ZnO Ag CNT	µg/L-range ng/L-range ng/L-range N/A	Indicative no effect concentrations (INECs) were established using an assessment factor of 1,000. The INECs are only given as orders of magnitude due to uncertainties. No specific number is given regarding number of studies used to derive the INECS.	"The data available so far are not sufficient to perform an absolute environmental risk assessment, especially due to the lack of both exposure and chronic effect data". "there are not enough data to discuss the long-term toxicity of realistic concentrations of ENM in the environment".	(Aschberger, Micheletti et al. 2011)
ZnO	2194 µg/L	No details given	-	(Johnson, Dumont et al.

ENM	PNEC value	Approach	Highlighted gaps	Reference
Ag	0.028 µg/L			2014)
TiO_2 ZnO Ag CNT nZVI	5.8 µg/L 0.042 µg/L 0.04 µg/L N/A N/A	Based on 22, seven and eight freshwater data, respectively, and an resulting AF of 1,000.	Need for more chronic toxicity studies (especially on daphnia and fish) for more reliable PNEC estimation.	(Stone, Hankin et al. 2010)

These PNEC values, the knowledge gaps related to the PNEC values and their estimation approaches will be discussed in Chapter 6 and compared to the PNEC values that are established based on the approach applied within this project.

3.5 Comparison of value ranges

Based on the information collected in Table 3, Table 4, Table 5 and Table 6 an overview of PNEC values for ionic, bulk and nano forms of the case study nanomaterials has been compiled (see Table 7). From this overview it is evident that the same PNEC values are often applied in REACH registration dossiers for the metal salts and the dossier of the corresponding metal or metal oxide bulk material. At the same time the dossiers for TiO_2, ZnO and Ag were stated to cover both nano and bulk forms, meaning that the bulk PNEC values also should apply to the nano-form of the substance included in the registration. The CuO dossier covers only the bulk form and no separate dossier is currently available for the nano form.

When comparing values from REACH registration dossiers with the PNEC values in the scientific literature it is noted that the literature PNEC values are generally lower compared to the values in the dossiers.

Table 7 - Overview of PNEC freshwater estimations obtained from ECHA/REACH or literature. All values are in µg/L.

Framework	TiO_2	ZnO	Ag	CNTs	CuO	nZVI	CeO_2	QDs	CB
ECHA database of registered substances									
Ionic[#]	-	20.6	0.04	-	7.8	-	-	-	-
Bulk	127	20.6	0.04	-	7.8	-	-	-	-
Nano	127	20.6	0.04	430-780	-	-	-	-	5.000-50.000
Literature									
Nano	1-61	0.042-2194	0.0007-1	40-60	0.48	8.3[*]	2-108	-	-

[#]: Corresponding metal salt; -: indicates that no data was identified or that the literature reported that PNEC values could not be established at present; [*]: predictions for long-term exposure.

From Table 7 it is evident that Ag nanoparticles have by far the lowest PNEC reaching a value of only 0.7 ng/L. This is in contrast to CB for which the PNEC is in the mg/L range. As can be seen from Table 7 there is a large variation in PNEC values for the selected case study ENMs depending on data source. For Ag nanoparticles the literature estimates range from 60 times lower to 25 times higher than the ECHA registrations, whereas for ZnO nanoparticles literature values are from almost 500 times lower to 100 times higher than the ECHA registrations. This variation is on one

hand due to some of the uncertainties and lack of data as highlighted above and thereby influenced of the choice of assessment factors which can change the resulting PNEC value by orders of magnitude.

On the other hand, the differences in the PNEC values for ENMs in Table 7 also reflect the importance of data selection for deriving PNEC values. This crucial step in the effect assessment is often difficult to evaluate and the literature review revealed an urgent need for a more transparent data selection and assessment of data adequacy for regulatory purposes. Prior to the estimation of PNEC values for ENMs is it therefore necessary to ensure that the data selected is indeed both reliable and relevant for this purpose. Since no specific guidelines for data selection of effect data for risk assessment purposes exist, it was found necessary to develop a set of evaluation criteria as will be described in Chapter 4.

4. A concept for evaluation of ecotoxicological data for ENMs

When evaluating available information on chemicals in the context of risk assessment under REACH the following elements are included in the assessment of data quality: reliability, relevance and adequacy. These terms have been defined by Klimisch, Andreae et al. (1997) as follows:

- **Reliability:** Evaluating the inherent quality of a test report or publication relating to preferably standardised methodology and the way the experimental procedure and results are described to give evidence of the clarity and plausibility of the findings.

- **Relevance:** Covering the extent to which data and tests are appropriate for a particular hazard identification or risk characterisation.

- **Adequacy:** Defining the usefulness of data for hazard/risk assessment purposes. Where there is more than one study for each endpoint, the greatest weight is attached to the studies that are the most relevant and reliable. For each endpoint, robust summaries need to be prepared for the key studies.

It is common practice that an ecotoxicological test result is considered more valid for regulatory use if it is obtained according to accepted and validated guidelines and even better if the laboratory conduct the study according to Good Laboratory Practice (GLP). Such accepted and validated guidelines could be international, e.g. ISO or OECD, or national e.g. DIN, ASTM or TNO, and they exist by the dozen. It is scientifically and ethically sound and reasonable to establish and follow such guidelines, as it will reduce costs and use of experimental organisms, thereby enabling different regulatory bodies to trust and accept previously derived ecotoxicological effect data according to the MAD principles. At the same time the use of standardised guidelines facilitates reproducibility of the test and comparability across substances (Ågerstrand, Breitholtz et al. 2011).

Klimisch, Andreae et al. (1997) developed a systematic approach to evaluate ecotoxicological effect data, with main emphasis on the extent to which the study followed guidelines and GLP. Today the Klimisch score is routinely applied to ecotoxicological studies submitted in technical dossiers to regulatory bodies by classifying them as reliable (K1-2) or not (K3-4). This has the purpose of assisting the assessor in the risk assessment procedure especially in the case where several (and potentially conflicting) data is available. The data relevance is subsequently assessed based on expert judgement in light of e.g. applicability of the data for the purpose of the specific hazard identification or risk assessment, environmental realism of testing strategy, consideration of test substance properties in the test design and usefulness of information obtained from tests on non-standard organisms. Based on reliability and relevance the study is then deemed adequate or not adequate for the specific regulatory purpose.

4.1 Guideline versus non-guideline test methodologies

The majority of current ecotoxicological test guidelines have been developed for testing of traditional chemicals, with a focus on narcotic and polar narcotic chemicals, where the test set-up is not expected to have critical influence on the speciation of the tested chemical. Reactive chemicals and specifically acting chemicals require more attention. For pharmaceuticals, for example, guideline testing may not be sufficiently sensitive and modified test systems (set-up as well as organisms) can provide valuable additional information to the risk assessment process (Ågerstrand, Breitholtz et al. 2011). Nevertheless, authorities still require pharmaceuticals tested according to guideline test methods. Similarly it can be argued that ecotoxicity data on ENMs produced using guideline tests may not be sufficiently sensitive. ENMs are known to behave very differently in ecotoxicity test systems compared to soluble chemicals, for which the guidelines were intended. However, nanoparticles do not dissolve in water; they form a suspension, agglomerate and/or release dissolved chemical species. This is the case for well-known ENMs like ZnO-NP, CuO-NP, and Ag-NP for which the relationship between the particulate and dissolved species of the metals remain a discussion when results of ecotoxicity studies are evaluated.

All of the ENM transformation processes that may occur before and during ecotoxicity testing may change the exposure in both qualitative and quantitative terms, see e.g. Hartmann, Skjolding et al. (2014). ENMs may thus have properties that are much different from traditional chemicals. Hence there is a risk of inducing test artefacts when applying these guidelines to ENMs. Currently established guidelines derived for testing of traditional chemicals thus need adaptation or re-design in order to make them suitable for testing ENMs. Work is ongoing within the OECD to develop nano-specific guidance but until then regulatory testing will continue to rely on existing test guidance. At present, however the use of guideline test methods, and thereby classification as K1 according to the Klimisch score, does not necessarily imply that the data is adequate for ERA of ENMs.

4.2 Evaluating the adequacy of ecotoxicity studies for ENMs

For the purpose of this work, a method was developed for assigning the adequacy of ecotoxicological studies of ENMs using a two-dimensional scoring system. A graphical illustration is shown in Figure 2. The method was developed based on an approach for evaluation of ecotoxicity data as developed and described by Moermond, Kase et al. (In prep). The first dimension is based on the method suggested by Moermond, Kase et al. (In prep) to assign a score for data reliability (Ri1-Ri4) but modified to include additional criteria for exposure characterisation in the test system, resulting in a modified 'nano-reliability' score (nRi1-nRi4). The second dimension relates to the relevance (Re1-Re4). The relevance scores and criteria are not considered to be different from those of conventional chemicals as they do not refer to the inherent properties of the test material or test system but rather address the data in the context of its use in e.g. risk assessment. We have therefore adopted the relevance criteria as proposed by Moermond, Kase et al. (In prep). The purpose was to develop a structured, transparent and reproducible approach to assist the expert judgement needed to assess the adequacy of ecotoxicological studies of ENMs for regulatory use. This approach aims at being applicable to evaluate both guideline and non-guideline studies. This represents a significant deviation from the currently used Klimisch score approach which inherently favours the use of guideline tests.

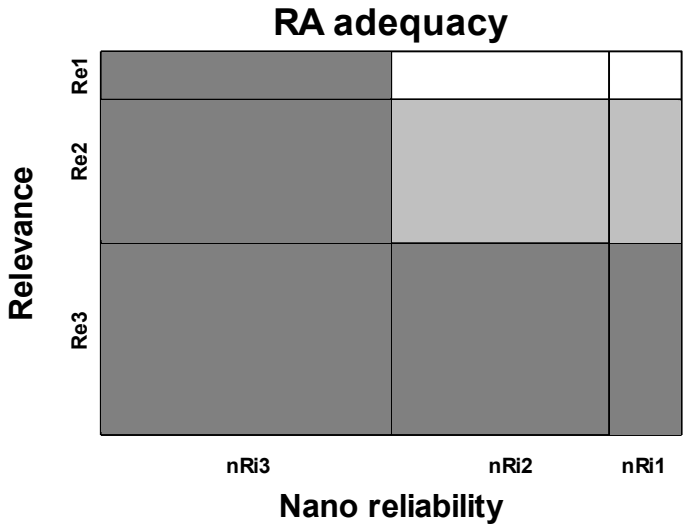


Figure 2 – Summary of the proposed two-dimensional approach for evaluation of the adequacy of ecotoxicity data in risk assessment of ENMs based on data reliability and data relevance. Adapted from Agerstrand, Kuester et al. (2011). White area represents studies "adequate for use for regulatory purposes", grey area represents studies "may be adequate for use for regulatory purposes" and dark grey area represents studies "not adequate for use for regulatory purposes".

4.2.1 Step 1: Reliability evaluation – documentation of experimental conditions and nanomaterial properties

When defining reliability criteria for ENM ecotoxicity data it is important to take into account the additional needs for data on physical and chemical properties as well as the characterization before and during testing. Nano-specific physico-chemical characteristics have been included in previously proposed methods to evaluate the quality of toxicological studies of ENMs (Card, Magnuson 2010). This method consists of two steps; step 1 on the general study relevance and reliability and step 2 on the characterisation data for the ENM. The general study reliability is assessed according to the Klimisch score (Klimisch, Andreae et al. 1997) by using the ToxRTool developed by Schneider, Schwarz et al. (2009). The 'nano-dimension' is assessed based on availability of information related to:

1. agglomeration and/or aggregation
2. chemical composition
3. crystal structure/crystallinity
4. particle size/size distribution
5. purity
6. shape
7. surface area
8. surface charge
9. surface chemistry (including composition and reactivity)
10. whether any characterization was conducted in the relevant experimental media.

The result of the evaluation in the approach by Card, Magnuson (2010) is expressed as a Klimisch score K4-1 indicating the general study reliability combined with the Nanomaterial score N1-10 indicating the availability of nanomaterial characterisation data. Combining these two individual scores results in a "nano study score" of KX-NY with K1-N10 being the best score and K4-N1 the worst in the sense of quality of the study. An ENM score of N5 is obtained if data for five of the listed 10 properties are provided along with the toxicological study. By incorporating the use of the

ToxRTool this approach is applicable to human toxicity data and thereby not applicable to ecotoxicity data.

Here we apply a similar approach by adding a 'nano-dimension' to the Klimisch score. The requirements to data on nano-specific characterisation includes the information required by Card, Magnuson (2010) but has been expanded to also address dynamic changes of the ENMs in the test systems. The major differences are that this approach:

- Is developed specifically for evaluation of *eco*toxicity data
- The results is one combined reliability score of nRi1-nRi4

The final score of nRi1-nRi4 gives an indication of the study reliability based on the documentation provided on the study design and data on inherent nanomaterial properties and nanomaterial characterization in the test system. We suggest a description of the nano-reliability scale for ecotoxicity studies of ENMs as listed in Table 8. The more extensive the documentation of general test conditions and nanomaterial characteristics, the higher an nRi-score can be assigned to the data.

Table 8 – Descriptions of a "nano-Reliability" score to describe the reliability of ecotoxicity studies of nanomaterials to be used for regulatory purposes.

Score	Description
nRi1	**Reliable without restrictions**: All critical (▲▲) and important (▲) reliability criteria are fulfilled. The study is well designed, performed and documented. Nanomaterial properties and behaviour in the test system is extensively documented. The experiment has been carried out according to methods that are considered scientifically appropriate for ecotoxicity testing of nanomaterials and where the physico-chemical properties of the nanomaterial are considered in the test design. If (when) specific nanomaterial guidance or guidelines exist, the use of these may be considered favourable.
nRi2	**Reliable with restrictions**: Most (>50%) critical (▲▲) and important (▲) criteria are fulfilled. The study is generally well designed, performed and documented, but some minor flaws in the documentation or setup may be present. Nanomaterial properties and behaviour in the test system is well documented. The experimental design and test method are considered scientifically appropriate for ecotoxicity testing of nanomaterials but may contain some minor flaws in documentation or setup.
nRi3	**Not reliable:** Not all critical reliability criteria are fulfilled. This mainly concerns studies which have clear flaws in study design, study conduction or reporting and/or where the experimental design and test method are considered not to be scientifically appropriate for ecotoxicity testing of nanomaterials.
nRi4	**Not assignable:** Information needed to make an assessment of the majority of the critical and important criteria is missing. This concerns studies or data from the literature which do not give sufficient experimental details and which are only listed in short abstracts or secondary literature (books, reviews, etc.), or studies or reports where the documentation is not sufficient for assessment of reliability for one or more critical parameters.

21 criteria were developed based on Moermond, Kase et al. (In prep) but modified to take into account the properties and behaviour of nanomaterials in aquatic test systems, see Table 9.

Guidance text was developed to support the data evaluation. Again, this guidance is based on Moermond, Kase et al. (In prep) but modified to take into account the properties and behaviour of nanomaterials in aquatic test systems. This guidance text can be found in appendix 3.

Table 9 – Reliability criteria for assigning a reliability score (nRi-nR4, see Table 8) to nano-ecotoxicity data. For a further explanation of the criteria, see the main text. The criteria have been divided in different levels of importance: critical, important, minor importance. The criteria are modified from Moermond, Kase et al. (In prep).

Number	Importance (▲▲/▲/△)	Criterion
		General information
		Before evaluating the test, check the physico-chemical characteristics of your compound (handbooks/general sources/scientific literature). What is the solubility of the nanomaterial? Is it coated? If yes, what are the known properties of the coating material? Is it photocatalytic or reactive?
1	▲▲	Is a sufficiently detailed description of endpoints and methodology available?
		Test setup
2	△	Is a standard method (e.g., OECD/ISO) or modified standard method used?
3	△	Is the test performed under GLP conditions?
4	(△/▲)	If applicable, are validity criteria fulfilled (e.g. survival or growth of controls)?
5	▲	Are appropriate controls performed (e.g. dispersant control, metal ion control, larger than nano-sized (bulk) particles of the same chemical composition, negative and positive control)?
		Test Compound
6	▲▲	Is the test substance appropriately identified with name or chemical identifier (e.g., CAS-number)? Are nanomaterial characteristics reported that allow for a clear identification of the tested material (e.g. particle size, shape, particle size distribution, surface area*)? Are test results reported for the appropriate compound?
7	▲	Is the purity of the test substance reported? This includes information on synthesis by-products as well as synthesis catalysts and presence of other crystalline forms of the substance. And/or, is the source of the test substance trustworthy?
8	(△/▲)	If a formulation is used or if impurities or coatings are present: Do other ingredients in the formulation, the impurities or the coatings exert an effect? Is the amount of test substance in the formulation known?
		Test Organism
9	▲	Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?
10	▲▲	Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms been pre-exposed to test compounds or other unintended stressors?
		Exposure Conditions
11	▲▲	Is the experimental test system, test design and test vessel scientifically appropriate for testing of nanomaterials (e.g., static, flow-through, renewal; light/dark conditions; open/closed systems; still/stirred; exposure route)?

Number	Importance (▲▲/▲/△)	Criterion
12	▲▲	Is the experimental system appropriate for the test organism (e.g., choice of medium or test water, feeding, water characteristics, temperature, light/dark conditions, pH, oxygen content)? Have conditions been stable during the test?
13	▲	Were exposure concentrations stable throughout the duration of the test (taking the use of a dispersant/stabilizer/solvent into account)? And was the exposure qualitatively stable? If not, has this been accounted for in the data interpretation? If a dispersant/stabilizer/solvent is used, is the dispersant/stabilizer/solvent within the appropriate concentration range and is a dispersant/stabilizer/solvent control included?
14	△	Is a correct spacing between exposure concentrations applied?
15	▲▲	Is the exposure duration defined?
16	▲▲	Have analyses been performed to verify exposure, e.g. substance concentrations and physico-chemical transformations of the test substance over the duration of the test?
17	▲	Is the biomass loading of the organisms in the test system within the appropriate range (e.g., < 1 g/L)?
		Statistical Design and Biological Response
18	▲	Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?
19	▲	Are appropriate statistical methods used?
20	△	Is a dose response curve observed? Is the response statistically significant?
21	▲	Is sufficient data available to check the calculation of endpoints and validity criteria (e.g., control data, dose-response curves)?

See explanatory guidance text on how to interpret these criteria in appendix 3. Please note that some criteria are not per se critical for the reliability of a study, and that this depends strongly on the compound and/or organisms tested.

▲▲ These criteria are critical for study reliability,

▲ These criteria are important for study reliability,

△ These criteria are of minor importance for study reliability, but may support study evaluation,

(△/▲) Importance of these criteria depend on the specific test and/or nanomaterial properties.

* For further details on minimum characterization requirements see explanatory guidance text.

As a pragmatic approach to determine and document the study evaluation a spreadsheet was constructed where criteria importance were assigned values of 1, 2 or 3 (corresponding to △, ▲ and ▲▲). The main reasons for using symbols and not just numbers were to stress the degree of criticality as well as the option for using the evaluation approach in a qualitative manner. However, when assigning the scores, the symbols had to be translated into numbers in order to calculate the final weighted score. Criteria fulfilment was ranked from 0-3, where 3: complete fulfilment, 2: partial fulfilment, 1: limited fulfilment, 0: criterion not addressed. By multiplying criteria importance with criteria fulfilment a sum was achieved for each criterion. After evaluating all 21 criteria a total reliability score for the study was determined. This is based on a calculation of % criteria fulfilment compared to the maximum achievable value as follows:

nRi1: Reliable without restrictions	Fulfilment/partial fulfilment of >90% of the criteria **AND** minimum partial fulfilment of **all** critical (▲▲) and important (▲) criteria
nRi2: Reliable with restrictions	Fulfilment/partial fulfilment of 61-90% of the criteria **AND** minimum partial fulfilment of >50% critical (▲▲) and important (▲) criteria
nRi3: Not reliable (supporting information)	Fulfilment of <60% of the criteria

The score nRi4 is given if there is insufficient data available to perform the evaluation – and/or according to the specific requirements stated in the guidance text (see Moermond, Kase et al. (In prep)).

4.2.2 Step 2: Relevance evaluation – applicability of data and test method

The relevance of data is evaluated based on the relevance of the resulting data in the context of the regulatory data-use (hazard identification or risk assessment). Therefore no nanomaterial-specific considerations are included in the relevance evaluation, and relevance criteria were therefore adapted from Moermond, Kase et al. (In prep) with the only addition of adding requirements on fulfilment of critical and important criteria. The description of the scores can be seen from Table 10.

Table 10 – Description of relevance scores (adapted from Moermond, Kase et al. (In prep).

Score	Description
Re1	**Relevant without restrictions:** Studies or data from the literature or reports which are relevant for the purpose for which the study is evaluated. All critical (▲▲) and important (▲) reliability criteria are fulfilled.
Re2	**Relevant with restrictions:** Studies or data from the literature or reports which have a limited relevance for the purpose for which the study is evaluated. Most (>50%) critical (▲▲) and important (▲) criteria are fulfilled.
Re3	**Not relevant**: Studies or data from the literature or reports which are not relevant for the purpose for which the study is evaluated.
Re4	**Not assignable:** Studies or data from the literature which do not give sufficient details and which are only listed in short abstracts or secondary literature (books, reviews, etc.), or studies or reports of which the documentation is not sufficient for assessment of relevance.

The criteria and guidance used in the evaluation process was the one described in Moermond, Kase et al. (In prep). However, we assigned different importance to the criteria (values of 1, 2 or 3 (corresponding to △: minor importance, ▲: important and ▲▲: critical), as seen in Table 11. This was done based on an expert judgement.

Table 11 – Relevance criteria for assigning a relevance score (Re1-Re4, see Table 10) to nano-ecotocicity data. For a further explanation of the criteria, see the main text. The criteria have been divided into different levels of importance: critical, important, minor importance. The criteria are adopted from Moermond, Kase et al. (In prep).

Number	Importance (▲▲/▲/△)	Criterion
		General information
		Before evaluating the test for relevance, check why you are evaluating this study. The relevance of the study might be different for different purposes (e.g., EQC derivation, PBT assessment, dossier evaluation for marketing authorization), also depending on the framework for which the evaluation is requested
		Biological relevance
1	▲▲	Is the organism tested relevant for the aquatic compartment?
2	▲	Is the organism tested relevant for the tested compound?
3	▲▲	Are the reported endpoints appropriate for the regulatory purpose?
4	▲▲	Are the reported endpoints appropriate for the investigated effects or the mode of action?
5	△	Is the effect relevant on a population level?
6	▲	Is the magnitude of effect statistically and biologically significant and relevant for the regulatory purpose (e.g. EC_{10}, EC_{50})?

Number	Importance (▲▲/▲/△)	Criterion
7	▲▲	Are appropriate life-stages studied?
8	▲▲	Are the experimental conditions relevant for the tested organism?
9	▲	Is the time of exposure relevant and appropriate for the studied endpoints and organism?
10	(△/▲)	If recovery is studied, is this relevant for the framework for which the study is evaluated?
		Exposure relevance
11	(△/▲▲)	In case of a formulation, other mixture, salts or transformation products: Is the substance tested representative and relevant for the substance being assessed?
12	▲	Is the tested exposure scenario relevant for the substance?
13	▲	Is the tested exposure scenario relevant for the organism?

See explanatory guidance text on how to interpret these criteria in Moermond, Kase et al. (In prep). Please note that some criteria are not per se critical for the relevance of a study, and that this depends strongly on the context of the use of the data.

▲▲ These criteria are critical for study relevance,

▲ These criteria are important for study relevance,

△ These criteria are of minor importance for study relevance, but may support study evaluation,

(△/▲) Importance of these criteria depend on the specific context.

Again, to determine and document the study evaluation a spreadsheet was constructed where criteria importance were assigned values of 1, 2 or 3 (corresponding to △, ▲ and ▲▲). Criteria fulfilment was ranked from 0-3, where 3: complete fulfilment, 2: partial fulfilment, 1: limited fulfilment, 0: criterion not addressed. By multiplying criteria importance with criteria fulfilment a sum was achieved for each criterion. After evaluating all 13 criteria a total relevance score for the study was determined. This is based on a calculation of % criteria fulfilment compared to maximum achievable value as follows:

Re1: Relevant without restrictions	Fulfilment/partial fulfilment of >90% of the criteria **AND** minimum partial fulfilment of **all** critical (▲▲) and important (▲) criteria
Re2: Relevant with restrictions	Fulfilment/partial fulfilment of 61-90% of the criteria AND minimum partial fulfilment of >50% critical (▲▲) and important (▲) criteria
Re3: Not relevant (supporting information)	Fulfilment of <60% of the criteria

The score Re4 is given if there is insufficient data available to perform the evaluation – and/or according to the specific requirements stated in the guidance text (see Moermond, Kase et al. (In prep)).

4.2.3 Step 3: Adequacy – combining reliability and relevance

By combining the evaluation of study reliability (Step 1) and study relevance (Step 2) the ecotoxicity studies may now be scored according to their adequacy for use for regulatory purposes, e.g. estimation of PNEC values. This process is described in Figure 2. As can be seen from the figure studies with reliability nRi3 and/or relevance score Re3 are considered not adequate (dark grey in Figure 2) for regulatory purposes. Studies with reliability nRi1 or nRi2 and relevance score Re1 or Re2 are either adequate (white in Figure 2) or may be adequate (grey in Figure 2). For studies that 'may be adequate for regulatory purposes' their use for e.g. PNEC estimation will have to be based on an expert judgement taking into account factors such as overall data availability or may be used as supplementary information.

4.3 Recommendations and limitations for PNEC estimation for ENMs

As also highlighted by Som et al. (2012) the "quality of published data is crucial for the process of risk assessment". This is true for both conventional and alternative approaches to PNEC estimation and risk characterisation.

In the former sections we have outlined the current methodologies for deriving PNEC values as well as outlining alternative approaches. The various benefits and drawbacks of the different approaches have been discussed. There has not been evidence for the need for developing a totally different approach for deriving PNEC values, in other words, the current methods may work just as well as a new concept. A general and underlying assumption is that solid and valid effect data for deriving proper PNEC values are available. We have furthermore discussed the challenges and obvious problems regarding the current framework for deriving PNEC values:

1) that effect studies are based on guidelines developed for soluble chemicals and therefore not suitable for nanomaterials and

2) that effect studies are assessed for their risk assessment adequacy according to the Klimisch score, which by nature favours studies conducted according to GLP and in accordance with (current) guidelines.

5. PNEC estimations for selected NMs

In this chapter PNEC estimations are provided for the nine selected ENMs. The first section gives a brief recap on the PNEC estimation methodology and implications for ENMs. The following nine sections present the ecotoxicological data, PNEC estimations as well as associated reservations and GAP analyses for each of the individual ENMs. For each ENM a graph illustrates the ecotoxicological studies' risk assessment adequacies assessed according to the developed framework presented in the preceding Chapter 4, see Figure 2. For each material a more detailed overview table is provided in appendix 1 showing which organisms have been tested, the endpoint, the effect concentration and the resulting risk assessment adequacy score. Finally, the last section gives an overview of all the estimated PNEC values, both in a tabulate manner, as well as in lyrics.

5.1 Method for PNEC estimations for the selected ENMs and implications thereof

PNEC estimations were established according to the REACH guidelines (see Chapter 2) using the ecotoxicological studies assessed to be most adequate, according to the assessment framework presented in Chapter 4.

The results of applying the developed framework are consistently and transparently assessed effect studies for their risk assessment adequacy. The result contains both an assessment of the relevance as well as the reliability of the study, seen in a nanomaterial perspective. This enables the selection of the current most solid effect study suitable for PNEC estimation and following risk assessment.

It has to be noted that the PNEC estimations are based on the currently available hazard data from the scientific literature. It can be expected that these evaluations may change in the future as new data emerge and as test methods are improved and revised to better address the challenges of testing ENMs.

Furthermore, that despite the good intentions of the applied AFs, they cannot correct for improperly conducted effect studies, or effect studies conducted according to guidelines not suited for nanomaterials. As long as the effect studies are not sufficiently reliable, this will have implications on the derived PNEC value and must be taken into consideration in the subsequent risk assessment.

PNEC estimations given in this Chapter are thus based on the assumptions that 1) the current test methods are applicable to nanomaterials, and 2) that the current extrapolation methods are valid for nanomaterials. Both of these assumptions are highly questionable (see Chapter 6). The values should therefore be taken as indicative for the order of magnitude for PNEC given the current regulatory recommendations for PNEC estimation and not be used as the definitive protective concentration for the environment.

5.2 Literature search strategy

In order to find scientific articles covering effect assessment of nanomaterials in relation to risk assessment, a literature search strategy was developed. Each of the selected nanomaterials (TiO_2, ZnO, Ag, CNTs, CuO, nZVI, CeO_2, QDs and CB) was combined with "tox*" and "nano*" and "alga*/crustacean*/daphni*/fish*" in Web of Science and resulted in 1,208 hits in total (as of 25 October 2014), see Table 12**Fejl! Henvisningskilde ikke fundet.**. A range of these articles were in fact replicates, as some articles were retrieved for the same ENM, but for different organisms, or because a single article reported data for several ENMs and therefore were retrieved for several ENMs. Across all searched nanomaterials and search strategies, there were 677 unique articles. By reading through the abstract and extracting important information from the methods and materials as well as the results sections of the articles, articles reporting some sort of median effect concentration (IC_{50}, LC_{50} and EC_{50}), some sort of no or low effect concentration (NOEC, LOEC, EC_{10} or EC_{20}), data on sub-acute, bioaccumulation, uptake, elimination or similar interesting information were retrieved, resulting in 500 articles.

Of this selection of relevant articles a further focused selection was done for the purpose of evaluating this focused selection according to the developed framework. As far as possible, proper attention was paid to include all studies reporting long-term NOEC-values on the three trophic levels of algae, daphnia and fish in respect of risk assessment of the aquatic environment. If no such articles were retrieved, articles reporting short-term EC_{50}-values were to the furthest extent included in the assessment. Less attention was paid to articles reporting some exotic effect on e.g. gene expression of immunotoxicity, however such studies have regularly been included in the assessment for risk assessment adequacy, despite their relative less relevance for risk assessment. This focused selection has resulted in articles being left out of the assessment, but to our best knowledge this will not significantly change the final output on PNEC estimation.

Table 12 – Overview of the literature search where each nanomaterial was combined with Tox* AND Nano* AND "alga*/crustacean*/daphni*/fish*" in Web of Science. Literature searches were performed till the end of October 2014.

	Alga*	Crustacean*	Daphni*	Fish*	**Total#**
Titanium dioxide	58	11	164	98	**257**
Zinc oxide	21	9	53	24	**77**
Silver	76	14	128	98	**262**
Carbon nanotubes	46	7	110	66	**181**
Copper oxide§	13	6	26	16	**50**
Iron (nZVI)	18	0	17	15	**44**
Cerium dioxide	3	2	10	4	**13**
Quantum dots	19	1	37	21	**65**
Carbon black	4	3	9	1	**14**
Sum	258	53	554	343	**963**

§: Copper oxide has been assessed instead of copper carbonate, as no ecotoxicological studies were found using the devised search strategy; #: The number of articles given in the "total column" is less than the sum of the articles from the four organisms. Similarly, summing up the number of articles from the "total column" is larger than the total retrieved articles. The reason for this is that one article can appear for several organisms as well as several ENMs and will therefore be counted for more than one.

From the amount of retrieved literature, as shown in Table 12**Fejl! Henvisningskilde ikke fundet.**, some interesting observations can be done. As expected, the most studied ENMs are silver, titanium dioxide and CNTs with around 200 articles for each ENM. In a middle group zinc oxide, copper oxide, nZVI and quantum dots appear with around 60 articles each. Finally, the least studied ENMs are cerium dioxide and carbon black.

Despite the anticipated vast literature on the application of biochar and soil amendments of some sort of activated carbon compounds, it is interesting to see that only 14 articles were retrieved

concerning nanosized carbon black in association with toxicity and the selected organisms. Maybe not that surprising, the amount of literature for quantum dots is limited, especially considering the wide variety of compositions quantum dots are made of. This limited amount of literature for both carbon black and quantum dots renders it doubtful whether a PNEC can be established for these two ENMs.

Generally, the most studied organism is the daphnia, which fits well with its status as a filter feeder, resulting in an anticipated higher exposure from settled ENMs compared with other organisms.

5.3 PNEC estimation for TiO_2 NPs

5.3.1 Overview and selection of key data based on a focused literature review

Around 20 articles reporting NOEC/EC_{10}-values including EC_{50} values were selected for further assessment, see Figure 3. Six studies were found adequate for regulatory use (white) and six studies were assessed as 'may be adequate for regulatory use (grey). A representative selection of these studies is displayed in Table 15 in the appendix, where it appears that no studies were assigned the highest possible adequacy score (nRi1-Re1). Again, the reason for the lower adequacy of some studies was the poor characterisation of the inherent properties of the tested nanomaterials. Even though several authors stated both particle size and ratio between anatase and rutile TiO_2 (e.g. Hartmann et al., 2010) it has not been possible to designate toxicity to either of the species. This is mainly due to the agglomeration and lack of characterization and exposure quantification during and after the test.

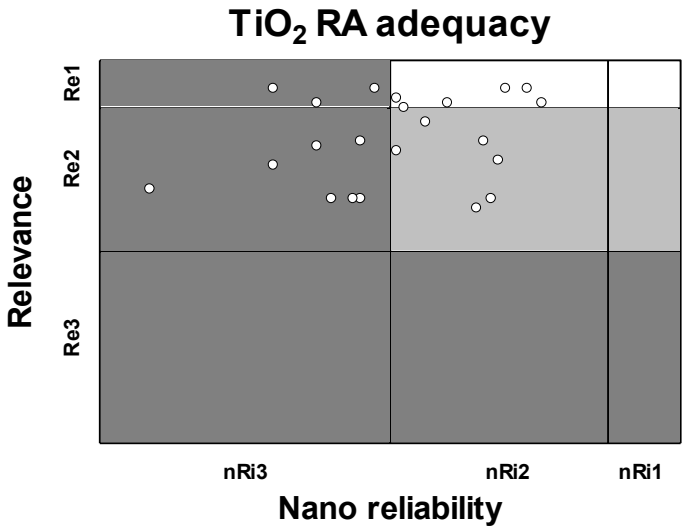


Figure 3 – Risk assessment adequacies of the effect studies of titanium dioxide (TiO_2) nanoparticles.

It appears that short term tests have been performed on all three trophic levels of algae, daphnia and fish. For algae, 72-h EC_{50} values are seen in the range of 2.53-241 mg/L (Lee, An 2013, Sadiq, Dalai et al. 2011, Hartmann, Von der Kammer et al. 2010). In 48-h immobilization studies of daphnia, LC_{50} values are found to be >10 and even >100 mg/L (Griffitt, Luo et al. 2008, Wiench, Wohlleben et al. 2009). Different kinds of short-term studies on fish have been performed revealing 48-h LC_{50} values >10 mg/L (Griffitt, Luo et al. 2008), 120-h LC_{50} values on embryos of 84 and >100 mg/L, depending on the composition of the tested TiO_2 (Vicario-Pares, Castanaga et al. 2014) and finally for embryo a 72-h LC_{50} value of >2,000 mg/L for a coated TiO_2 ENM (Felix, Ortega et al. 2013). Among the tested organism the algae appear to be the most sensitive on a short-term basis, with an EC_{50} of 2.53 mg/L (Lee, An 2013). Chronic studies have been performed on both algae and

daphnia revealing 72-h NOEC like data from <0.5 to 3.3 mg/L (Lee, An 2013, Sadiq, Dalai et al. 2011, Hartmann, Von der Kammer et al. 2010) on algae. For daphnia, only one study obtained an EC_{10} value on cumulative offspring of 5.02 mg/L (Wiench, Wohlleben et al. 2009). For details see Table 15 in the appendix.

As compared with the silver nanoparticles, for which more 'exotic' endpoints of malformations, gene expressions and enzyme effects were tested (see section above), the more usual endpoints of mortality, growth and number of offsprings have been tested for TiO_2. Also the concentration levels causing effects is much higher for TiO_2, compared with for example silver. For TiO_2 several tests did not show effects at 10 and even 100 mg/L TiO_2. However, for titanium dioxide it seems that algae are the most sensitive organisms. The effect concentrations for daphnia and fish are in the mg/L range, whereas the no effect concentration levels on algae are in the upper µg/L range.

5.3.2 PNEC for TiO_2 nanoparticles

As data from short-term tests are available for all three base-set test organisms as well as some long-term chronic data on the most sensitive of the short-term tests, an assessment factor of less than 1,000 can be applied. Chronic test revealing NOEC values have been performed on both daphnia and algae allowing an assessment factor of 50.

An EC_{10}-value of 5.02 mg/L (Wiench, Wohlleben et al. 2009) was obtained for the chronic effects on offspring production from *D. magna*. The most sensitive test result was observed in the 72-h growth inhibition test with *P. subcapitata* with a NOEC of <0.5 mg/L (Lee, An 2013). However, since this study only report that NOEC is less than 0.5 mg/L and no LOEC is given, the study is not usable for PNEC estimation. Instead the estimation will have to be made using the 72-h growth inhibition NOEC of 0.89 mg/L for *Chlorella spp.* (Sadiq, Dalai et al. 2011).

A $PNEC_{freshwater}$ value of 18 µg/L can therefore be derived for titanium dioxide considering the reservations mentioned in the beginning of this chapter.

The following considerations are performed as a kind of sensitivity analysis. An assessment factor of 50 was set, based on the availability of adequate data. However, if the study used for the PNEC estimation should not be the defining study, the study by Hartmann, Von der Kammer et al. (2010) gives a 72-h growth inhibition EC_{10}-value of 3.3 mg/L resulting in a PNEC value of 66 µg/L.

As not enough NOEC values across enough taxa could be found, there is no option for SSD modelling.

Not enough or no data were available to derive PNEC values for WWTP, air, soil/terrestrial, sediment or the marine environment.

5.3.3 Knowledge gaps and uncertainties

- Titanium dioxide is generally found to be **non-toxic up to several mg/L** within the range of traditional hazard assessment studies on algae, daphnia and fish, though **no long term study on fish** could be found and a few **algae studies showed toxicities in the high µg/L range**.

- More studies across environmental compartments should be conducted.

- The most important reservation is that it was shown that **titanium dioxide nanoparticles agglomerate** during the duration of the ecotoxicity test performed, e.g. Sadiq, Dalai et al. (2011), Hartmann, Von der Kammer et al. (2010), and are to some

extent photolabile/photoreactive, thereby **hampering the prerequisite of a steady exposure** during the duration of the test (Hartmann, Skjolding et al. 2014) for further explanation.

- The studies already performed were not all adequate for regulatory risk assessment, which was mainly due to the **general lack of material characterisation**, especially measurements of the dose metric during and at the end of the test, but in fact also for basic data on inherent properties, e.g. for material identification and characterisation.

- Mechanistic studies of the mode of action on the nanoparticle as well as environmental fate processes are desired.

5.4 PNEC estimation for ZnO NPs

5.4.1 Overview and selection of key data based on a focused literature review

Of the retrieved articles for ZnO, 18 reported IC/LC/EC_{50} or NOEC/LOEC/EC_{10} data of mainly acute, but also a few chronic, effects, relevant for regulatory purposes. No study obtained the most adequate assessment of nRi1-Re1, but still five studies were found "adequate for risk assessment" (white) and six studies were assessed as "may be adequate for risk assessment" (grey). The remaining seven studies were found "not adequate for risk assessment" (dark grey), see Figure 4. No studies followed GLP, but most studies were performed according to some OECD or ISO standard, but the main reason for low reliability was the lack of characterization and especially the lack of exposure quantification throughout the test duration.

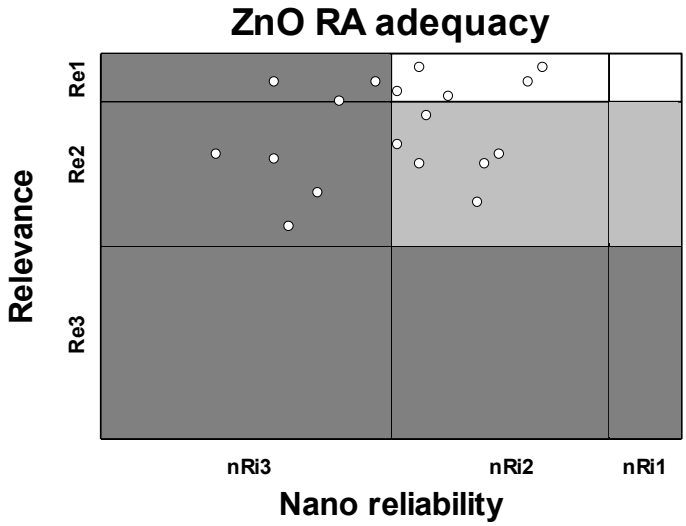


Figure 4 – Risk assessment adequacies of the environmental effect studies of zinc oxide (ZnO) nanoparticles.

Core ZnO nanoparticles, including a range of different coatings, were tested. The studies comprised nominal particle diameters from 20-100 nm, if at all stated. A range of studies reported agglomeration of the particles during the time course of the test. Table 16 in the appendix states the nominal particle sizes, when reported in the articles. Despite some articles reported similar particle sizes, it was not possible to ensure that identical particles were tested, as hardly any CAS#s were reported. Nevertheless, in the following analysis and discussion of the results, it is anticipated that identical materials were tested throughout the articles.

Some articles not only reported effects for the nano-form, but also for the bulk-form and/or the ionic Zn^{2+} form. In most cases the ionic form was more toxic and often the nano-form was at least as toxic as the bulk-form.

The far majority of the studies were dealing with freshwater. Only Jarvis, Miller et al. (2013) addressed two marine organisms (algae and daphnia), however their study was assessed to be "not adequate for risk assessment" with the score nRi3-Re2 (dark grey). Two studies addressed soil bacterial organisms; (Rousk, Ackermann et al. 2012) was assessed "not adequate for risk assessment" (with some EC_{50} values for bacterial growth inhibition of >5 g ZnO per g soil). Pokhrel, Silva et al. (2012) obtained the grey assessment (nRi2-Re2) and could be used as supporting information for the risk assessment process. However, the inhibition of the enzyme β-galactosidase endpoint is not directly applicable to risk assessment, as follows from the grey score, and an EC_{50} value of 80 mg/L were found for organic coated ZnO particles. One white study (Gladis, Eggert et al. 2010) (nRi2-Re1) was performed on surface-living algae, where ZnO was tested for its antifouling properties with or without UV irradiation. After UV irradiation a chronic NOEC of 0.06 mg/cm² was obtained.

For the freshwater compartment white studies (nRi2-Re1) covered short-term acute tests at all three trophic levels of the base-set, with EC_{50} for *P. subcapitata* <0.5 mg/L (Lee, An 2013), LC_{50} for *D. magna* of 1-100 mg/L (Wiench, Wohlleben et al. 2009) and LC_{50} for embryos of *D. rerio* of 1589 mg/L (Felix, Ortega et al. 2013). Additionally chronic studies of the algae and daphnia were performed with a NOAEC for *P. subcapitata* <0.5 mg/L (Lee, An 2013) and a NOEC for *D. magna* of 0.125 mg/L (Lopes, Ribeiro et al. 2014).

Additionally grey (nRi2-Re2) cell viability studies for the fish *P. lucida* and the protozoa *T. thermophilia* were performed, revealing NOEC values of 2-25 mg/L (Luisa Fernandez-Cruz, Lammel et al. 2013) and EC_{50} values of 4-8 mg/L (Mortimer, Kasemets et al. 2010), respectively.

5.4.2 PNEC for ZnO nanoparticles

As data from short-term tests are available for all three base-set test organisms as well as some chronic data, an assessment factor of less than 1,000 can be applied. At the short-term acute level, the algae is the most sensitive organism. As chronic NOEC data have been derived for both the algae and the daphnia, an assessment factor of 50 can be applied to derive the PNEC value.

The reported growth inhibition NOEC value for *P. subcapitata* <0.5 mg/L (Lee, An 2013) and the reproduction NOEC for *D. magna* is 0.125 mg/L (Lopes, Ribeiro et al. 2014).

A $PNEC_{freshwater}$ value of 2.5 µg/L can therefore be derived for zinc oxide nanoparticles considering the reservations mentioned in the beginning of this chapter.

The following considerations are performed as a kind of sensitivity analysis. An assessment factor of 50 was set, based on the availability of adequate data. However, if the study used for the PNEC estimation should not be the defining study, another study with an effect data only a factor of 4 higher would be used resulting in a PNEC value of 10 µg/L.

Not enough NOEC values from enough different taxa could be found and therefore the SSD modelling cannot be applied.

Not enough or no data were available to derive PNEC values for WWTP, air, soil/terrestrial, sediment or the marine environment.

5.4.3 Knowledge gaps and uncertainties

- More studies across environmental compartments should be conducted, as well as long-term studies on fish. Toxicities were generally found in the low mg/L range, with a few exceptions for daphnia, where toxicity was found in the high µg/L range. It was often reported, that the ionic form of zinc was more toxic than the bulk- and the nano-form, but a few times the nano-form was more toxic than the bulk-form, indicating specific nano effects.

- The most important reservation is that, despite all studies reported data for zinc oxide nanoparticles, the individual **particle size** claimed or determined are **not the same** for all assessed studies. Despite no big difference were observed based on particle size, this difference most likely affects the effect exerted by the zinc oxide nanoparticles, if maintained during exposure. Similarly, **coatings** may either enhance or prohibit ecotoxicological effects, e.g. increased absorption or decreased release of ions.

- Additional, zinc oxide nanoparticles were often shown to agglomerate during the test performance, thus **hampering the prerequisite of a steady exposure** (Hartmann, Skjolding et al. 2014).

- The studies already performed were not all adequate for regulatory risk assessment, which was mainly due to the **general lack of material characterisation**, especially measurements of the dose metric during and at the end of the test, but in fact also for basic data on inherent properties, e.g. for material identification and characterisation.

- Mechanistic studies of the mode of action on the nanoparticle as well as environmental fate processes are desired.

5.5 PNEC estimation for Ag NPs

5.5.1 Overview and selection of key data based on a focused literature review

No long term studies reporting chronic NOEC values were identified through our literature search, however, a number of studies reported significant long-term effects on reproduction and/or growth (Zhao, Wang 2011, Blinova, Niskanen et al. 2013, Pokhrel, Dubey 2012). As seen from Table 17 in the appendix, no studies were assigned the highest possible adequacy score (nRi1-Re1). Only two studies Georgantzopoulou, Balachandran et al. (2013) and Gaiser, Fernandes et al. (2012) (nRi2-Re1) were found to be adequate for regulatory use (white), despite Gaiser, Fernandes et al. (2012) did not have a conventional endpoint, but only reported 56% mortality at the tested concentration of 100 µg/L. Three studies Griffitt, Luo et al. (2008), Navarro, Piccapietra et al. (2008) and Gaiser, Fernandes et al. (2012) (nRi2-Re2) were assessed as 'may be adequate for regulatory use' (grey) and the rest were found not to be regulatory adequate (dark grey), because of very limited characterisation of the inherent properties of the studied nanomaterial resulting in an nRi3 classification of the reliability. Figure 5 illustrates how the risk assessment adequacies of the different selected studies are distributed based on the undertaken assessment of the adequacy.

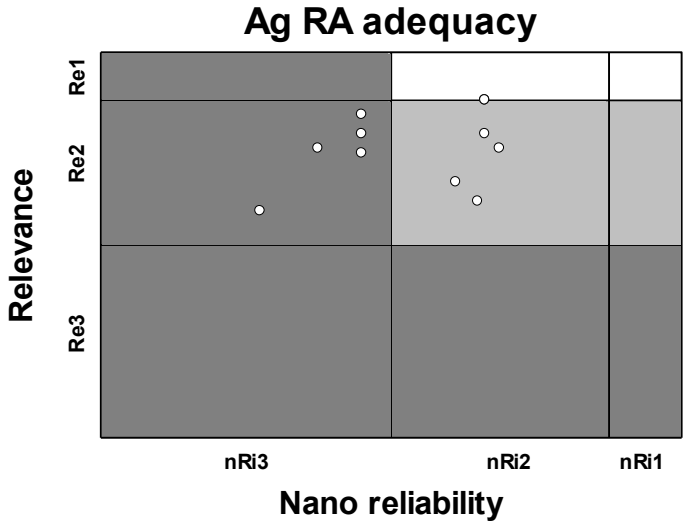


Figure 5 – Risk assessment adequacies of the environmental effect studies of silver (Ag) nanoparticles.

Core Ag nanoparticles, including a range of different coatings, were tested. The studies comprised nominal particle diameters from 20-100 nm, if at all stated. A range of studies reported agglomeration of the particles during the time course of the test. Table 17 in the appendix states the nominal particle sizes, when reported in the articles. Despite some articles reported similar particle sizes, it was not possible to ensure that identical particles were tested, as hardly any CAS#s were reported. Nevertheless, in the following analysis and discussion of the results, it is anticipated that identical materials were tested throughout the articles.

Some articles not only reported effects for the nano-form, but also for the bulk-form and/or the ionic Ag^+ form. In most cases the ionic form was more toxic and often the nano-form was at least as toxic as the bulk-form.

Among the studies scored as not adequate for regulatory purposes were two articles investigating the effects on fish embryos. While being relevant for sub-acute effect assessment, the reliability of these studies was not sufficient. Yeo, Kang (2008) studied different morphological, enzymatical and genetical effects of two test concentrations by stating the differences from the control group, whereas Asharani, Wu et al. (2008) studied similar effects, however, using a range of test concentrations. For similar endpoints, e.g. edema, notochord, heart rate, a factor of seven orders of magnitude was seen in the concentration to obtain the same level of effect.

Navarro, Piccapietra et al. (2008) studied the effects on photosynthesis of algae over a 5-hour period of time and though the study involved an extensive characterization, the effects are to be considered as acute effects. In the algal tests used for regulatory decision making a chronic endpoint is used (reproduction) and therefore this paper was evaluated as not adequate for regulatory risk assessment.

This leaves four studies adequate for risk assessment: Georgantzopoulou, Balachandran et al. (2013) and Gaiser, Fernandes et al. (2012) (nRi2-Re1) and Griffitt, Luo et al. (2008) and Gaiser, Biswas et al. (2011) (nRi2-Re2). Two of the studies include results from short-term tests reporting on chronic effects on algae with EC_{50}-values of 34 µg/L (72 hours) and 190 µg/L (96 hours). The same two studies also include short-term 48-hour acute effects on daphnia with EC_{50} values of 1.2 and 40 µg/L for adults, respectively, and 67 µg/L for neonates (Griffitt, Luo et al. 2008). Gaiser, Fernandes et al. (2012) also reports a short-term 96 hours 56% mortality at 100 µg/L for daphnia. The results from Griffitt, Luo et al. (2008) shows one order of magnitude less sensitivity compared

to the Georgantzopoulou, Balachandran et al. (2013) study and Gaiser, Fernandes et al. (2012) are even two orders of magnitude less sensitive. Georgantzopoulou, Balachandran et al. (2013) also studied the effects on bacteria with a luminescence EC_{50} of 420 µg/L and Griffitt, Luo et al. (2008) studied the 48-hour mortality on fish with an LC_{50} of 7,070 µg/L for adults and 7,200 µg/L for juveniles.

Gaiser, Biswas et al. (2011) studied the chronic survival of daphnia and reported 0-30% mortality at 1-50 µg/L, though no full dose response curve was shown and the effect was not dose dependent (non-monotone). Along with the EC_{50} value, Georgantzopoulou, Balachandran et al. (2013) also reported an EC_{10} value of 10 µg/L for the short-term chronic growth inhibition on algae.

5.5.2 PNEC estimation for Ag nanoparticles

Not only short-term data on each of the three base-set test organisms exists and, hence, an AF of less than 1,000 can be applied. However, solid long-term NOEC values could only be found for algae as the long-term studies on daphnia do not use adequate endpoints and the effect level is not unambiguous. Nevertheless, long-term studies exist for both algae and daphnia, indicating that an AF of 50 can be applied. The long-term $EC_{0\text{-}30}$ values for the daphnia (*D. magna*) by Gaiser, Biswas et al. (2011) are at the level of 1-50 µg/L. A few other studies support the finding of low or close to no chronic effects around 1-5 µg/L for *D. magna* (Zhao, Wang 2011, Pokhrel, Dubey 2012) justifying the lower AF. The short-term chronic EC_{10} value for the algae *D. subspicatus* is 10 µg/L. However, the lowest effect value among the base-set test organisms originates from the Georgantzopoulou, Balachandran et al. (2013) acute study on daphnia with an EC_{50}-value of 1.2 µg/L. This means that an AF of 100 must be applied resulting in a **$PNEC_{freshwater}$ of 12 ng/L** considering the reservations mentioned in the beginning of this chapter.

The following considerations are performed as a kind of sensitivity analysis. If the long-term $EC_{0\text{-}30}$ values of 1-50 µ/L for the daphnia (Gaiser, Biswas et al. 2011) are accepted along with the short-term chronic EC_{10} value for algae of 10 µg/L, an AF of 50 can be applied resulting in a $PNEC_{freshwater}$ of 20 ng/L. If the suggested study for PNEC estimation by Gaiser, Biswas et al. (2011) is not the defining study, there are other studies with effects in the same range, e.g. Zhao, Wang (2011) resulting in a $PNEC_{freshwater}$ of 100 ng/L instead. However, if the not traditional chronic endpoints on daphnia are not accepted as adequate, an AF of 1,000 must be applied to the acute study on daphnia with the EC_{50} value of 1.2 µg/L (Georgantzopoulou, Balachandran et al. 2013), resulting in a $PNEC_{freshwater}$ value of 1.2 ng/L. Again, if the study by Georgantzopoulou, Balachandran et al. (2013) used for the PNEC estimation should not be the defining study, an effect value one order of magnitude higher would have been the defining value; 40 µg/L for daphnia by Griffitt, Luo et al. (2008), resulting in a $PNEC_{freshwater}$ of 40 ng/L.

As not enough NOEC values across enough taxa could be found, there is no option for SSD modelling.

Not enough or no data were available to derive PNEC values for WWTP, air, soil/terrestrial, sediment or the marine environment.

5.5.3 Knowledge gaps and uncertainties

- Mainly short-term ecotoxicity studies were found for nanosilver, emphasising the **need for more chronic studies** in order to refine the hazard assessment, especially across environmental compartments. Nevertheless, effects were found in the µg/L-range.

- The most important reservation is that, despite all studies reported data for silver nanoparticles, the individual **particle size** claimed or determined are **not the same** for

all assessed studies, also because of agglomeration (Hartmann, Skjolding et al. 2014). This difference in particle size most likely affects the effect exerted by the silver nanoparticles, because one of the main purposes for manufacturing the nano form, is the enhanced reactivity proportional to the smaller size. Similarly, **coatings** may either enhance or prohibit ecotoxicological effects, e.g. increased absorption or decreased release of ions.

- The studies already performed were not all adequate for regulatory risk assessment, which was mainly due to the **general lack of material characterisation**, especially measurements of the dose metric during and at the end of the test, but in fact also for basic data on inherent properties, e.g. for material identification and characterisation.

- Mechanistic studies of the mode of action on the nanoparticle as well as environmental fate processes are desired.

5.6 PNEC estimation for Carbon Nanotubes (CNTs)

5.6.1 Overview and selection of key data based on a focused literature review

A total of 20 articles were found to report ecotoxicological information for CNTs and of these 15 were selected for further assessment due to their reporting of IC_{50}, LC_{50}, EC_{50}, EC_{10} or NOEC data or other relevant toxicological information. The remaining articles reported either cytotoxicity or appeared to be reviews. Figure 6 gives an overview of the risk assessment adequacy of the 15 articles and more detailed information about the toxicological endpoints, test duration, organisms and concentrations are shown in Table 18 in the appendix for a selection of the studies. Ten studies were assessed as 'may be adequate for regulatory use (grey). As seen from Table 18 in the appendix, no studies were assigned the highest possible adequacy score (nRi1-Re1), and were not even assessed white (adequate for risk assessment). The remaining five studies got a dark grey evaluation, meaning "not adequate for risk assessment".

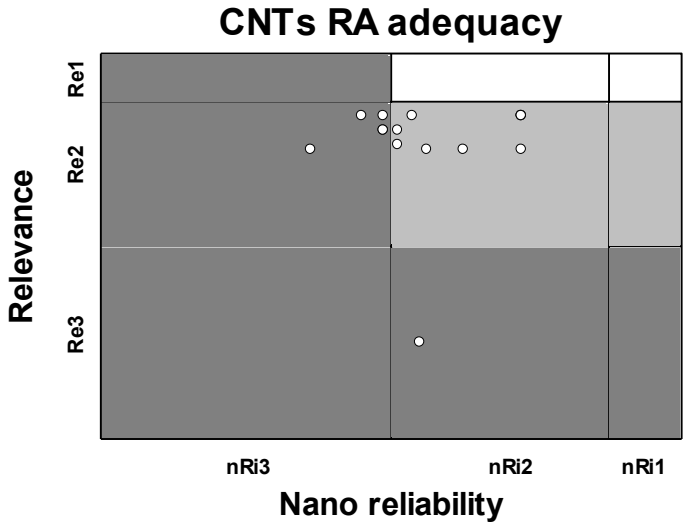


Figure 6 – Risk assessment adequacies of the environmental effect studies of carbon nanotubes (CNTs) nanoparticles.

Core CNT nanoparticles were tested. The studies comprised nominal particle diameters from <10-100 nm, if at all stated. A range of studies reported agglomeration of the particles during the time course of the test. Table 18 in the appendix states the nominal particle sizes reported in the selected articles. Despite some articles reported more or less similar particle sizes, it was not possible to ensure that identical particles were tested, as hardly any CAS#s were reported, and also different

experimental conditions were used. Nevertheless, in the following analysis and discussion of the results, it is anticipated that identical materials were tested throughout the articles.

If including the study on fish embryos by Asharani, Serina et al. (2008) (dark grey area on Figure 6), all three trophic levels of the base-set organisms have been tested. However, no traditional 96-h LC_{50} on fish or 48-h LC_{50} on daphnia were reported, but 96-h EC_{50} on growth inhibition on algae was reported. The EC_{50} values were reported in the range of 1.8-63 mg/L (Edgington, Roberts et al. 2010, Asharani, Serina et al. 2008, Schwab, Bucheli et al. 2011, Long, Ji et al. 2012). Two chronic studies on algae and daphnia revealed a NOEC for the algae of 0.042 mg/L (Schwab, Bucheli et al. 2011) and a significantly reduced reproduction for the daphnia at 0.125 mg/L (Edgington, Roberts et al. 2010).

5.6.2 PNEC for CNT

Provided that exposure times deviating slightly from guideline standards are accepted in the data selections and that the fish study scored as "dark grey" (Asharani, Serina et al. 2008) is accepted as well, data from short-term tests are available for all three base-set test organisms. Furthermore, if "a significant effect on daphnia reproduction" (i.e. the effect was different from the control group) is accepted as an analogue to a LOEC value (which may be divided by two to obtain an estimate for a NOEC), also chronic data on algae and daphnia are available, resulting in use of 50 as the assessment factor for PNEC estimation. The reported growth inhibition NOEC value for *C. vulgaris* is 0.042 mg/L (Schwab, Bucheli et al. 2011) and the estimated reproduction NOEC for *C. dubia* is 0.0625 mg/L (Edgington, Roberts et al. 2010).

A $PNEC_{freshwater}$ value of 0.84 µg/L can therefore be derived for carbon nanotubes considering the reservations mentioned in the beginning of this chapter.

PNEC wise, the most critical parameter is the lack of regulatory adequate studies. **Strictly following the guidelines, it is not possible to derive a PNEC value, as no proper studies are available!** Violating the assumptions, as described above, makes it possible to apply an assessment factor of 50, however, on very loose grounds. It would however be most appropriate to require more studies to be carried out.

The dataset for CNTs does not comprise enough NOEC values from enough different taxa and therefore the SSD modelling approach cannot be applied.

Not enough or no data were available to derive PNEC values for WWTP, air, soil/terrestrial, sediment or the marine environment.

5.6.3 Knowledge gaps and uncertainties

- Carbon nanotubes were shown to be toxic in the mg/L range, however, a few studies showed toxicity in the lower µg/L range. **In general there is a lack of regulatory adequate studies, and thus a need for relevant and reliable studies of both acute and chronic character**.

- More studies across environmental compartments should be conducted.

- The studies already performed were not adequate for regulatory risk assessment, which was mainly due to the **general lack of material characterisation**, especially measurements of the dose metric during and at the end of the test, but in fact also for basic data on inherent properties, e.g. for material identification and characterisation. Also basic

test parameters like validity criteria and controls, as well as observation of dose-response curves were lacking.

5.7 PNEC estimation for CuO NPs

5.7.1 Overview and selection of key data based on a focused literature review

Of the retrieved articles for CuO, 16 reported IC/LC/EC_{50} or NOEC/LOEC/EC_{10} data of mainly acute, but also a few chronic, effects, relevant for regulatory purposes. As can be seen from Table 19 in the appendix, no study was assigned the highest possible adequacy score (nRi1-nRe1) and only one study was found adequate for regulatory use (white) and nine studies were assessed as 'may be adequate for regulatory use' (grey). The main reason that several of the studies have been assigned with lower adequacy (grey) is the reliability of the studies. For most of the tests, the reliability is affected by the fact that the tests are not conducted following a standardized method or that the tests are not performed under GLP conditions as well as giving little or no characterization and quantification data of the ENMs. Figure 7 illustrates how the risk assessment adequacies of the different studies are based on the undertaken assessment of the adequacy.

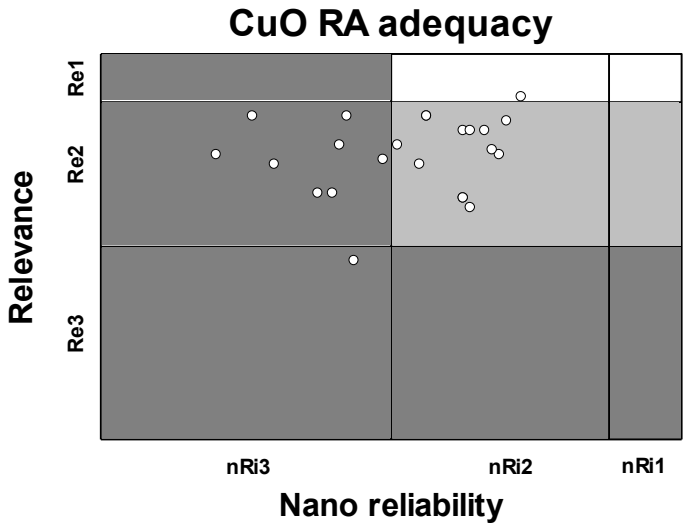


Figure 7 – Risk assessment adequacies of the environmental effect studies of copper oxide (CuO) nanoparticles.

Short-term ecotoxicity tests have been conducted for all three trophic levels of algae, daphnia and fish and based on these tests, daphnia are the most sensitive of the test organisms. Tests towards algae showed a 72-h EC_{50} value of 150 mg/L for *C. reinhardtii* (Melegari, Perreault et al. 2013) and a 96-h EC_{50} value of 0.54 mg/L for *P. subcapitata* (Griffitt, Luo et al. 2008). For daphnia the 48-h LC_{50} ranged from 0.034-0.42 mg/L, dependent on organism, neonate/adult and ENM (Kennedy, Melby et al. 2013, Griffitt, Luo et al. 2008, Rossetto, Vicentini et al. 2014). 48-h and 120-h acute tests on the fish *D. rerio* (embryos, juveniles and adults) showed LC_{50} values in the range 0.71-1.56 mg/L (Griffitt, Weil et al. 2007, Griffitt, Luo et al. 2008), with one study >10 mg/L (Vicario-Pares, Castanaga et al. 2014).

Chronic NOEC values have been published for both algae and daphnia, where daphnia also show higher sensitivity than algae. A 72-h NOEC value of ≤100 mg/L was observed for *C. reinhardtii* (Melegari, Perreault et al. 2013). However, based on EC_{50} values, *P. subcapitata* is more sensitive than *C. reinhardtii*, but no NOEC value was obtained for this organism. The one study that was assessed white (adequate for risk assessment) (Rossetto, Vicentini et al. 2014) (nRi2, Re1), studied the long-term chronic effects on daphnia (*D. magna*) resulting in NOEC values of 0.06 mg/L

(mortality and reproduction as endpoints) and <0.01 mg/L with growth as endpoint. The study also included a 15-min acute test with bacteria (*V. fischeri*), resulting in an EC_{50}-value of 7.79 mg/L (see Table 19 in the appendix (Rossetto, Vicentini et al. 2014)).

In all of the reviewed studies where a metal salt ($CuCl_2$ or $CuSO_4$) has been included as a reference for free ion toxicity, the metal salt showed greater toxicity compared with the ENM. In general, the toxicity of Cu-compounds can be ranged in the following order: bulk CuO<CuO NP<Cu^{2+} based on the results from the reviewed studies (Kasemets, Suppi et al. 2013, Blinova, Ivask et al. 2010, Heinlaan, Ivask et al. 2008, Aruoja, Dubourguier et al. 2009, Mortimer, Kasemets et al. 2010, Vicario-Pares, Castanaga et al. 2014, Rossetto, Vicentini et al. 2014). Griffitt, Luo et al. (2008) also found that Cu^{2+} was more toxic than CuO nanoparticles, except for juvenile zebrafish, where the LC_{50} for CuO nanoparticles was 2.5 times lower than for Cu^{2+}.

All of the abovementioned studies have been conducted in artificial or natural freshwater. The literature search resulted in only three studies where the test organism (all three studies have used the deposit-feeding snail *P. antipodarum*) have been exposed through sediment (Pang, Selck et al. 2012, Pang, Selck et al. 2013, Ramskov, Selck et al. 2014). Exposure to different forms of Cu (e.g. soluble Cu, nano-CuO, and micro-CuO) resulted in no or minimal mortality of the test organisms (Ramskov, Selck et al. 2014, Pang, Selck et al. 2012). However, exposure to different forms of Cu affected specific growth rate and feeding rates, and the effect on these two endpoints was significantly enhanced for nano-CuO compared to aqueous Cu (Pang, Selck et al. 2012).

5.7.2 PNEC for CuO nanoparticles

As data from short-term tests are available for all three base-set test organisms as well as some chronic data, an assessment factor of less than 1,000 can be applied. At the short-term acute level, the daphnia is the most sensitive organism. As chronic NOEC data have been derived for both the daphnia and algae, an assessment factor of 50 can be applied to derive the PNEC value. The reported growth inhibition NOEC value for *C. reinhardtii* was ≤100 mg/L (Melegari, Perreault et al. 2013) and the mortality, reproduction and growth NOEC values for *D. magna* is 0.06, 0.06 and <0.01 mg/L, respectively (Rossetto, Vicentini et al. 2014). However, the short-term LC_{50} value for *C. dubia* of 0.034 mg/L (Kennedy, Melby et al. 2013) represents the lowest effect value, resulting in the use of an AF of 100.

A $PNEC_{freshwater}$ value of 0.34 µg/L can therefore be derived for copper oxide considering the reservations mentioned in the beginning of this chapter.

The following considerations are performed as a kind of sensitivity analysis. An assessment factor of 100 was set, based on the availability of adequate data. However, if the study used for the PNEC estimation should not be the defining study, an assessment factor of 50 had to be applied on a daphnia reproduction NOEC value (as this is now the lowest effect data) of 0.06 mg/L resulting in a PNEC value of 1.2 µg/L.

Not enough NOEC values from enough different taxa could be found and therefore the SSD modelling cannot be applied.

Not enough or no adequate data were available to derive PNEC values for WWTP, air, soil/terrestrial, sediment or the marine environment.

5.7.3 Knowledge gaps and uncertainties

- More studies across environmental compartments should be conducted, as well as long-term studies on algae and fish. Toxicities were generally found in the µg/L range, with **more or less equal toxicity across the base-set trophic levels**.

- It was generally reported, that the ionic form of copper was more toxic than the nano form, but the nano form was always more toxic than the bulk form, indicating specific nano effects.

- The most important reservation is that, despite all studies reported data for copper oxide nanoparticles, the **media composition** and especially pH are **not the same** for all assessed studies. This will inevitable have an effect on the obtained effect data.

- The studies already performed were not adequate for regulatory risk assessment, except for one. The main reason for this level of reliability was the **lack of stable and quantifiable exposure conditions**.

- Mechanistic studies of the mode of action on the nanoparticle as well as environmental fate processes are desired.

5.8 PNEC estimation for nano Zero Valent Iron (nZVI)

5.8.1 Overview and selection of key data based on a focused literature review

In the search strategy the words "iron" and "nano" were used in order to make sure that all studies, including studies on nano Zero Valent Iron (nZVI) were included in the results. Out of the 50 hits in the literature search, 7 studies were with nZVI as a test compound. The other studies were primarily with nano-Fe_2O_3 or Fe_3O_4 as test compounds. Out of the seven studies with nZVI as test compound, two studies were reporting EC_{50} or LOEC-values. Additionally, six studies with other iron-based nanomaterials as test compounds were reporting IC_{50}, LC_{50}, EC_{50} or NOEC/LOEC, and these are also included in the assessment in order to increase the amount of data to support the PNEC estimations.

As can be seen from Table 20 in the appendix, no study was assigned the highest possible adequacy score (nRi1-Re1). Two studies were found adequate for regulatory use (white) and three studies were assessed as 'may be adequate for regulatory use' (grey). The main reason for a lower reliability score is the lack of verification of exposure and exposure conditions during the tests and lack of appropriate controls (e.g. larger than nano-sized controls or whether coatings or other ingredients in the test formulations exert an effect), which have not been included in a large number of the studies.

Figure 8 illustrates how the risk assessment adequacies of the different studies are assessed based on the undertaken assessment of the adequacy.

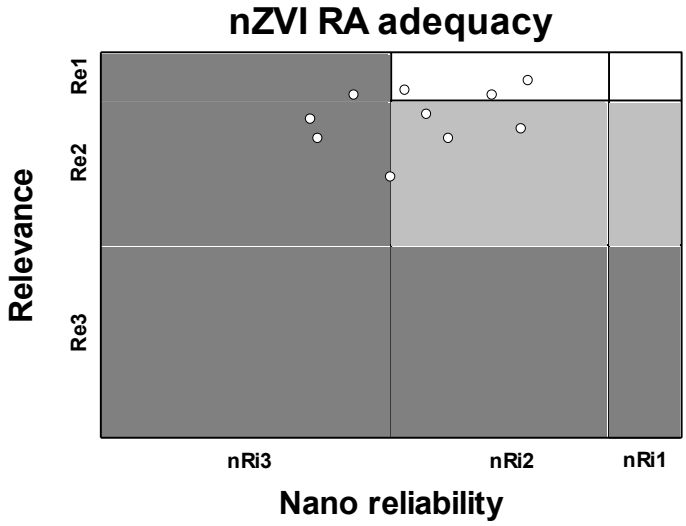


Figure 8 – Risk assessment adequacies of the environmental effect studies of iron containing (nZVI/Fe_2O_3) nanoparticles.

Varying sorts of acute short-term ecotoxicity tests have been carried out for all three trophic levels of algae, daphnia and fish. Chronic LOEC values have also been obtained for algae. According to the short-term tests daphnia is the most sensitive test organisms. However, test with the marine algae *T. pseudonana* show that this specific organism is very sensitive to nZVI (Keller, Garner et al. 2012) (nRi2, Re1).

Two studies were assessed to be adequate for risk assessment (assigned in the white area, see Table 20 in the appendix). Marsalek, Jancula et al. (2012) (nRi2, Re1) investigated the toxicity of zero-valent iron nanoparticles against several aquatic organisms (algae, daphnia, fish, bacteria and plants). The test material was a commercially available product (Nanofer 25), which contains >90% Fe^0 in the solid phase (with FeO and Fe_3O_4 as impurities). The results showed that nZVI was very toxic towards cyanobacteria (EC_{50} = 50 mg/L). The toxicity of nZVI towards the tested organisms was in the following order: cyanobacteria>aquatic plant>algae>daphnia>fish>terrestrial plant. No exact EC_{50} values were provided. It was however mentioned that for daphnia the EC_{50} is > 1,000 mg/L) and for fish EC_{50} > 2,500 mg/L. Keller, Garner et al. (2012) (nRi2, Re1) also investigated the 96-h toxicity of three commercially available products (Nanofer 25S and Nanofer 25 (suspensions) and Nanofer STAR (nanopowder)) and dissolved iron (Fe^{2+} and Fe^{3+}) towards different marine and freshwater microalgae and the freshwater crustacean (*D. magna*). In all cases Nanofer 25S was the most toxic of the three test compounds, and also more toxic than dissolved iron, except towards *P. subcapitata*, where a growth LOEC (Fe^{2+}) was found to be 5 mg/L compared to 8.24 mg/L for the Nanofer 25S. The LOEC values towards the marine organisms were much lower, ranging from 0.42-3.1 mg/L. For *D. magna*, the survival LOEC was 0.5 mg/L.

The literature search did not identify any studies in the soil or sediment compartments. Keller, Garner et al. (2012) investigated the effect of nZVI on three marine algae organisms and found that especially one organism (*T. pseudonana*) showed high sensitivity when exposed to nZVI suspensions in seawater (NOEC = 0.42 mg/L).

5.8.2 PNEC for nZVI

Before PNEC estimation is carried out, it is important to realize that nZVI will react immediately after application and will under aerobic conditions change physical and chemical form to larger than nano-sized particles consisting of iron-oxides. Therefore, a traditional PNEC for the aquatic

environment (based on tests carried out under aerobic conditions) is more or less meaningless. Given the short-lived nature of nZVI, it may be more sensible to apply the principles for deriving an intermittent PNEC, where an assessment factor of 100 is applied to the lowest acute EC_{50} value (ECHA 2008).

However, according to our literature search, the acute short-term data found do not report the traditional endpoints, i.e. either the duration or the quantification of the endpoint is different. Provided that the present data are treated as traditional effect data, the lowest acute effect data is a 96-h daphnia survival test resulting in a LOEC of 0.5 mg/L (Keller, Garner et al. 2012).

A $PNEC_{freshwater,\ intermittent}$ value of 5 µg/L can therefore be derived for nano zero valent iron for this type of exposure considering the reservations mentioned in the beginning of this chapter.

The following considerations are performed as a kind of sensitivity analysis. An assessment factor of 100 was set, based on the availability of data and the nature of the nanoparticle. However, if the study used for the PNEC estimation should not be the defining study, another study with a similar effect data would have to be used, not changing the out coming of the PNEC value drastically.

The dataset for nZVI does not comprise enough NOEC values from enough different taxa and therefore the SSD modelling cannot be applied.

Not enough or no adequate data were available to derive PNEC values for WWTP, air, soil/terrestrial, sediment or the marine environment.

5.8.3 Knowledge gaps and uncertainties

- There is a general lack of, especially chronic, toxicity data for nZVI across environmental compartments. Mechanistic studies of the mode of action of the nanoparticle as well as environmental fate processes are desired for risk assessment.

- Despite all studies reported data for nZVI, the individual **particle size** claimed or determined are **not the same** for all assessed studies. Additionally, the ZVI nanoparticles contain varying degrees of reactive iron species responsible for the toxicity.

- The studies were not all adequate for regulatory risk assessment, which was mainly due to the **general lack of material characterisation**, especially measurements of the dose metric during and at the end of the test, but in fact also for basic data on inherent properties, e.g. for material identification and characterisation.

- The nanoparticles were often shown to agglomerate during the test and as they also rapidly oxidize in contact with water **the prerequisite of a steady exposure in the tests are not fulfilled**.

- As bare nZVI tend to agglomerate within minutes and quickly oxidize when released, unintended environmental contact with ZVI nanoparticles are not expected. It is **unclear what a long-term PNEC value for ZVI nanoparticles signify** when the particles only have a very brief lifetime in the environment.

5.9 PNEC estimation for CeO_2 NPs

5.9.1 Overview and selection of key data based on a focused literature review

Of the 19 hits in the literature search, 16 studies have reported IC_{50}, LC_{50}, EC_{50} or NOEC/LOEC data or other toxicological information. The risk assessment adequacy evaluation was performed as described earlier, see Figure 9. As can be seen from Table 21 in the appendix, no study was assessed with the highest grade for risk assessment adequacy, but two studies were found in the "white" area and eight studies were found in the "grey" area. The main reason that most of the studies have been assigned within the grey area is the reliability of the studies, which is greatly affected by a lack of verification of exposure and exposure conditions during the tests, which have been general for most of the assessed studies with CeO_2 nanomaterials. Detailed information about the toxicological endpoints, test duration, organisms and concentrations are shown in Table 21 in the appendix for a selection of the studies.

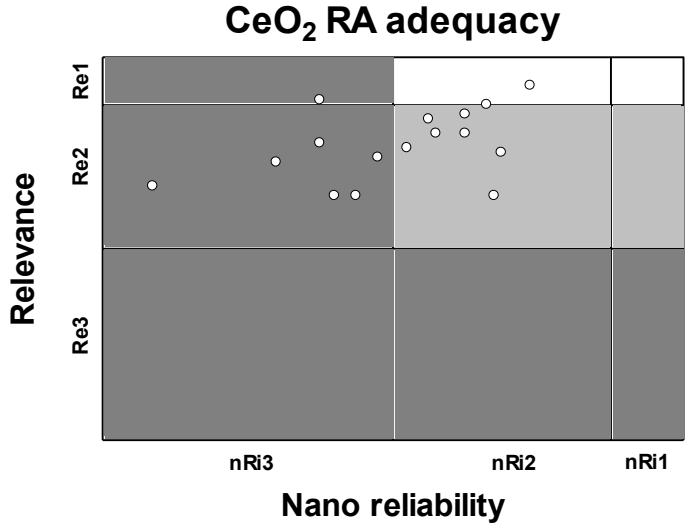


Figure 9 – Risk assessment adequacies of the environmental effect studies of cerium dioxide (CeO_2) nanoparticles.

Short-term ecotoxicity tests have been carried out for all three trophic levels of algae, daphnia and fish. The resulting toxicity values are, however, very scattered. Chronic EC_{10}/NOEC values have been published for both daphnia and algae, with algae showing higher sensitivity than daphnia.

Two studies were assessed to be adequate for risk assessment (assigned white, see Table 21 in the appendix). Felix, Ortega et al. (2013) investigated the toxic effect of CeO_2 nanoparticles coated with a polymer coating consisting of poly(acrylic acid) (PAA) towards zebrafish embryos on different endpoints and found a LC_{50}-value of > 2,000 mg/L (Felix, Ortega et al. 2013) (nRi2, Re2). This corresponds well with the results from Van Hoecke, Quik et al. (2009) (nRi2, Re2), who found EC_{50} values for test with the same test organism of > 200 mg/L (i.e. the highest test concentration). Gaiser, Fernandes et al. (2012) (nRi2, Re1) carried out acute and chronic exposures of *D. magna*, and found no acute toxicity of CeO_2 nanoparticles up to the highest tested concentration (10 mg/L). Up until 10 mg/L no chronic toxicity was observed either, however, 100% mortality was found in the 10 mg/L test concentration after 7 days exposure.

However, the results from Artells, Issartel et al. (2013) (nRi2, Re2) suggests high intra organism sensitivity and implies that *D. magna* may not be the most sensitive organism of crustaceans as an EC_{50}-value of 0.26 mg/L was found for *D. similis*.

Van Hoecke, Quik et al. (2009) (nRi2, Re2) performed acute and chronic tests on organisms at three trophic levels and used these results to derive PNEC values of 0.052 mg/L, 0.068mg/L, and 0.108 mg/L for 14 nm, 20 nm, and 29 nm CeO_2 nanoparticles, respectively. The PNEC values were estimated by the AF approach using an AF of 50 as described in Table 6. However, as exposure concentrations were not verified throughout the experiment, these results should be assessed with some caution.

Of the 16 studies selected for review, seven studies investigated the toxicity of micro-sized CeO_2 in parallel with the tests with the nanoparticles and in all cases nano-CeO_2 was found to be more toxic than the micro-sized particles (Van Hoecke, Quik et al. 2009, Manier, Bado-Nilles et al. 2013, Tomilina, Gremyachikh et al. 2011, Gaiser, Biswas et al. 2011, Gaiser, Fernandes et al. 2012, Rodea-Palomares, Boltes et al. 2011, Rogers, Franklin et al. 2010). Several studies have also investigated the dissolution from CeO_2 nanoparticles and found that the particles were basically insoluble and toxicity is thus not assumed to be caused by the dissolved fraction (Van Hoecke, Quik et al. 2009, Felix, Ortega et al. 2013, Gaiser, Biswas et al. 2011, Manier, Bado-Nilles et al. 2013, Rogers, Franklin et al. 2010). The toxicity of CeO_2 nanoparticles towards aquatic organisms is therefore assumed to be due to direct physical interaction between the particles and the organism, possible causing membrane disruption (Manier, Bado-Nilles et al. 2013, Rogers, Franklin et al. 2010). The adhesion of particles to both daphnia and algae have been confirmed in several studies (Gaiser, Biswas et al. 2011, Artells, Issartel et al. 2013, Manier, Bado-Nilles et al. 2013, Rodea-Palomares, Boltes et al. 2011, Rogers, Franklin et al. 2010).

With regards to sub-lethal effects Gaiser, Biswas et al. (2011) found a significant effect on moulting in *D. magna* after 96 h exposure to 10 mg/L nano-CeO_2 compared to the control. Significant effects on animal growth at lowest test concentration (0.01 mg/L) and in the highest test concentration (10 mg/L) compared to control was also found. DNA damage (primarily DNA strand breaks) was furthermore observed in *D. magna* and *C. riparius* after 96 hours exposure to 15 and 45 nm CeO_2 particles (Lee, Kim et al. 2009).

The literature search only resulted in one study on terrestrial organisms, namely a test with the soil organisms *C. elegans* (Roh, Park et al. 2010). The *C. elegans* tests were conducted in nematode growth medium (NGM) agar and not in actual soil. The endpoints were growth, fertility, mortality and stress-response gene expression. Most of the tested genes were not significantly changed by CeO_2 nanoparticle exposure, only the expression of cyp35a2 was increased compared to control. The biological function of this gene in *C. elegans* is, however, still unknown. A 20% decrease in survival rate compared to control was found for organisms exposed to 7 nm CeO_2 particles, but not for 45 nm particles. Also fertility was affected, as a 28 and 11% decrease in the number of eggs per worm was found compared to control after exposure to 15 and 45 nm CeO_2 particles, respectively. No effect on animal growth was found (Roh, Park et al. 2010).

5.9.2 PNEC for CeO_2 nanoparticles

As data from short-term tests are available for all three base-set test organisms as well as some chronic data, an assessment factor of less than 1,000 can be applied. At the short-term level, the daphnia is the most sensitive organism with an LC_{50} value of 0.26 mg/L (Artells, Issartel et al. 2013). As chronic NOEC data have been derived for both the algae and the daphnia, an assessment factor of 50 can be applied to derive the PNEC value. The reported growth inhibition EC_{10} value for *P. subcapitata* was 0.7 mg/L (Manier, Bado-Nilles et al. 2013) and the reproduction EC_{10} for *D. magna* was 8.8 mg/L (Van Hoecke, Quik et al. 2009). The EC_{10}-values are used as surrogates for NOEC values.

Using the short-term EC_{10} value of 0.7 mg/L for algae a $PNEC_{freshwater}$ value of 14 µg/L is derived, however, in this case, the tested daphnia organisms on the acute (*D. similis*) and the chronic (*D. magna*) levels are not the same. As the organism tested at the acute level is much more sensitive

than the organism tested at the chronic level, it is expected that a chronic test result for the most sensitive organism at the acute level, would result in a much lower chronic NOEC as reported above. In fact, the acute LC_{50} value for the most sensitive organism is even lower than the short-term EC_{10} value for the algae and it is therefore justified to use the acute LC_{50} value for daphnia of 0.26 mg/L, resulting in a **$PNEC_{freshwater}$ value of 5.2 µg/L** for cerium dioxide nanoparticles considering the reservations mentioned in the beginning of this chapter.

The following considerations are performed as a kind of sensitivity analysis. An assessment factor of 50 was set, based on the availability of adequate data. However, if the study used for the PNEC estimation should not be the defining study, the algae study would have to be applied an assessment factor of 100 resulting in a PNEC value of 7 µg/L, similar to the derived PNEC-value.

Not enough NOEC values from a sufficient number of different taxa could be found and therefore SSD modelling cannot be performed.

The data availability, or lack of same, is not sufficient to derive PNEC values for WWTP, air, soil/terrestrial, sediment or the marine environment.

5.9.3 Knowledge gaps and uncertainties

- Cerium dioxide is generally found to be **non-toxic up to several mg/L** within the range of traditional hazard assessment studies on algae, daphnia and **fish embryos** and a few **algae and daphnia studies showed toxicities in the medium-to-high µg/L range**.

- More studies across environmental compartments should be conducted.

- The most important reservation is that it was shown that **cerium dioxide nanoparticles agglomerates** during the duration of the ecotoxicity test performed, thereby **hampering the prerequisite of a steady exposure** during the duration of the test, see Hartmann, Skjolding et al. (2014) for further explanation.

- The studies already performed were not all adequate for regulatory risk assessment, which was mainly due to the **lack of exposure quantification** , especially measurements of the dose metric during and at the end of the test, but in fact also for basic data on inherent properties, e.g. for material identification and characterisation.

- Mechanistic studies of the mode of action on the nanoparticle as well as environmental fate processes are desired.

5.10 PNEC estimation for Quantum Dots (QDs)

5.10.1 Overview and selection of key data based on a focused literature review

Of the 65 hits in the literature search a range of the articles reported data on bioaccumulation, transformation and other environmentally related fate parameters including cytotoxicity and immunotoxicity. Six articles were found to contain risk assessment relevant ecotoxicological information, where five articles reported LC_{50} or EC_{50} values, see Table 22 in the appendix. The six relevant articles were further assessed according to the principles described earlier. Two of the six studies, Bouldin, Ingle et al. (2008) and Contreras, Cho et al. (2013), obtained a score of nRi3-Re2 for the risk assessment adequacy, i.e. in the “dark grey” area in Figure 10). The other four studies, Pokhrel, Silva et al. (2012), Wang, Zhang et al. (2008), Lee, Kim et al. (2009) and Kim, Park et al. (2010) obtained nRi2-Re2, i.e. in the “grey” area for risk assessment adequacy (Figure 10). For the

aquatic compartment only algae and daphnia were studied and for the terrestrial compartment a bacteria and a nematode were studied.

The relevance of the studies for risk assessment was generally high, except for the bacteria study mainly due to a questionable relevance of the end point used. However, the reliability was generally on a lower level (especially for the nematode study and one of the algae studies), where especially identification and quantification of the nanoparticles and relevant controls and validity criteria lacked. Figure 10 illustrates how the risk assessment adequacies of the different studies are based on the undertaken assessment of the adequacy. Further details of the studies are found in Table 22 in the appendix.

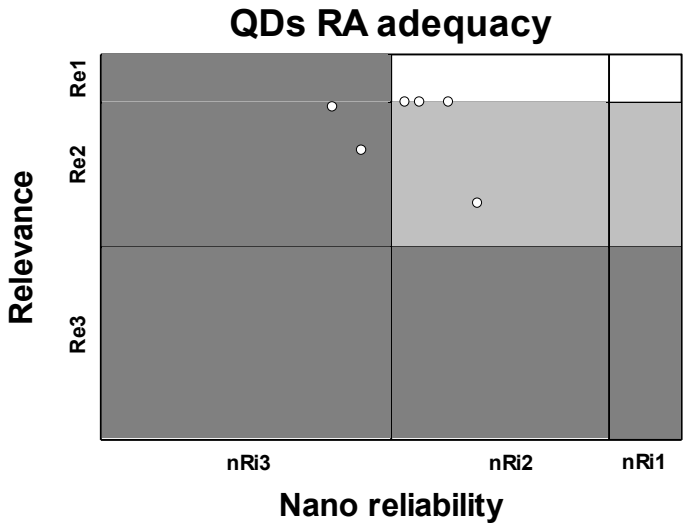


Figure 10 – Risk assessment adequacies of the environmental effect studies of quantum dots (QDs) nanoparticles.

Based on the MetPLATE™ bioassay, measuring the β-galactosidase inhibition of *E. coli* in the terrestrial compartment, up to 100 mg/L of the octadecylamine coated Cd/Se-QD nanomaterial tested only exerted a 34% inhibition (Pokhrel, Silva et al. 2012); Risk assessment adequacy score: nRi2-Re2, grey).

For the freshwater compartment the study by Wang, Zhang et al. (2008) (Risk assessment adequacy score: nRi2-Re2, grey) revealed a short-term EC_{50}-value on the algae *C. reinhardtii* of 5 mg/L and a significant effect from 0.1 mg/L towards stress response genes. The two other studies by Lee, Kim et al. (2009) and Kim, Park et al. (2010), from the same research group, (risk assessment adequacy score: nRi2-Re2, grey), both report on effects from irradiation activation of different coatings of the Cd/Se-QDs on *D. magna*. The exposure level was quantified in terms of Cd^{2+} for comparison with a Cd-salt, which in all cases were more toxic than the nanoparticles. Except for the mercaptopropionic acid coating and white fluorescence irradiation giving an EC_{50}-value larger than 2.5 mg/L, all 48-h EC_{50} mortalities ranged from 0.01-0.4 mg/L, dependent on coating and light conditions, with irradiation by sunlight and gum arabicum coating as the most toxic (Lee, Kim et al. 2009). Sub-lethal effects were reported down to 0.003 mg/L for significant effects on mRNA.

No data were found for marine waters, sediment, WWTP or air.

5.10.2 PNEC for Quantum Dots

This limited dataset leaves no option for SSD modelling for QD nanoparticles. Given the very limited data set, the low diversity of organisms tested (two algae organisms, two crustacean

organisms, one nematode and a bacteria) one organism of crustaceans, molluscs, and insects) and the regulatory adequacy of the studies, we find that it is not justifiable to apply the assessment factor approach for PNEC derivation. Furthermore, the studies reported do not test the same material and therefore the set of ecotoxicity data is even more limited than indicated above. Thus, no PNEC can be estimated for QD nanoparticles at the present state-of-knowledge.

5.10.3 Knowledge gaps and uncertainties

QD nanoparticles are semiconducting nanocrystals. Due to their unique physical, chemical and optical properties, they have a wide and increasing application range within the solar cell industry, light-emitting devices and biological and medical imaging (Lee, Kim et al. 2009, Kim, Park et al. 2010). QDs are not unique nanoparticles in the sense that they constitute different core and core-shell materials, e.g. CdSe, CdSe/ZnS and CdTe/CdS. Additionally they are mostly coated with a variety of organic coatings, e.g. mercaptoproprionic acid, gum arabicum, octadecylamine. Because of the increased application range, the likelihood of environmental releases is increasing, resulting in an increased risk for the environment.

The ecotoxicity of QDs have not been extensively studied and the studies that have been conducted have a medium reliability, mainly due to lack of exposure quantification but medium-high relevance. Mainly studies on *D. magna* for different coatings and under different irradiation conditions have been performed. All studied QDs are cadmium containing and since Cd is a highly ecotoxic metal even a low degree of leaching of Cd from the QD may give rise to significant effects.

There is a general lack of studies, making it difficult to firmly assess the level of toxicity. More studies are recommended both on a wider range of organisms (not even the base-set organisms have been studied), but also with respect to the identification of the QD nanomaterial, e.g. surface coating. This regards both acute as well as chronic studies and for different compartments, i.e. no data on marine or sediment organisms were available. More studies are needed on the same material in order to perform a proper PNEC-estimation.

5.11 PNEC estimation for Carbon Black (CB)

5.11.1 Overview and selection of key data based on a focused literature review

Of the 14 hits in the literature search six articles were found to contain relevant ecotoxicological information. However, only two articles have reported LC_{50} or EC_{50} values Mesaric, Sepcic et al. (2013) and Rodd, Creighton et al. (2014) and three reported sub-lethal effects Rodd, Creighton et al. (2014, Canesi, Ciacci et al. (2008) and Liu, Vinson et al. (2009). The four relevant articles were further assessed according to the principles described earlier (Chapter 4), see Table 23 in the appendix. The two EC_{50}/LC_{50}-studies and one of the sub-lethal effect studies were performed on marine organisms all reporting acute effects (Mesaric, Sepcic et al. 2013, Rodd, Creighton et al. 2014) and the remaining sub-lethal effect study was performed on a terrestrial organism reporting chronic effects (Liu, Vinson et al. 2009). The studies by (Mesaric, Sepcic et al. 2013, Canesi, Ciacci et al. 2008, Liu, Vinson et al. 2009) were assessed as nRi3-Re2, whereas the study by (Rodd, Creighton et al. 2014) reporting LC_{50}-values and effects on HSP70 for marine crustacean larvae (*A. franciscana*) was the only study with an nRi3-Re1 score. However, all four studies were assessed dark grey. All studies lacked nanomaterial characterization and exposure evaluation and this is the main reason for the low regulatory reliability assessment. Figure 11 illustrates how the risk assessment adequacies of the different studies are based on the undertaken assessment of the adequacy. Further details of the studies are found in Table 23 in the appendix.

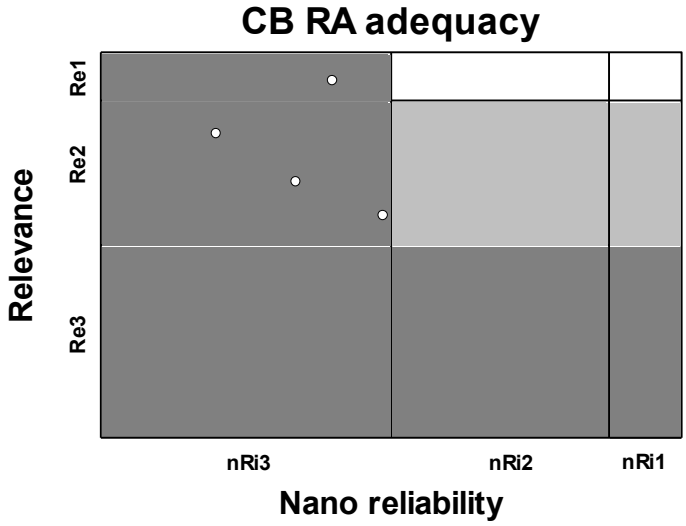


Figure 11 – Risk assessment adequacies of the environmental effect studies of carbon black (CB) nanoparticles.

Rodd, Creighton et al. (2014) studied CB with three different surfaces and it was found that the commercially produced had the lowest LC_{50}-value of 370 mg/L, whereas the two surface modified particles (more and less hydrophobic) were less toxic with LC_{50}-values of 1,000 and >1,000 mg/L, respectively. For the sub-lethal effects on increased HSP70 production both the commercially produced and the more hydrophobic showed significant effects already at 50 mg/L, whereas the less hydrophobic did not show significant effects.

Liu, Vinson et al. (2009) performed studies on a fly and did not find significant effect on hatching from 1,000 µg CB per g food, however, exposure of the adult fly to 3.3 mg in a vial of unknown volume showed significant effects on survival.

No data were found for freshwater, sediment, WWTP or air.

5.11.2 PNEC for CB

This limited dataset leaves no option for SSD modelling. Given the very limited data set and the diversity of organisms tested (one organism of crustaceans, molluscs, and insects) it is at present not possible to apply the assessment factor approach for PNEC derivation. Thus, no PNEC can be estimated for CB nanoparticles at the present state-of-knowledge.

5.11.3 Knowledge gaps and uncertainties

CB nanoparticles are used for soil amendments, fertilising, water purification and in some cases it can be used for detoxification of humans in the form of activated carbon. CB is not a uniquely characterised product and therefore exists in many particle sizes as well as with varying elemental compositions, i.e. different ratios among carbon, hydrogen and oxygen. There is thus a direct intentional and unintentional exposure to the environment, but not with well characterised particles.

The toxicity of CB has not been extensively studied with regards to its ecotoxicological effects and the studies that have been conducted have a low reliability for regulatory decision support, due to lack of characterisation. However, a range of sub-lethal effects were found significant at a level of 1 mg/L in the marine mussel. Similar EC_{50}/LC_{50} studies (with low regulatory reliability) showed effects in the high mg/L to low g/L range towards a marine crustacean. The general lack of studies makes it difficult to firmly assess the level of ecotoxicity of CB. More studies need to be carried out

on a wider range of organisms. This applies to acute as well as chronic studies and for different compartments. Thus, it is critical for PNEC estimation that data on freshwater base set organisms becomes available.

5.12 Overview of derived PNEC values for the selected ENMs

In Table 13 an overview of the derived PNEC values are shown. It was not possible to derive PNEC values for CB and QDs due to the very limited data set for these two ENMs. For the rest of the ENMs the data availability allowed the use of an AF of 50, except for nZVI nanoparticles which were treated as particles with an intermittent release, where an AF of 100 is applied the lowest acute effect data and except for Ag and CuO, where an AF of 100 also was applied due to a short-term effect value being the lowest effect value. For the long-term PNECs it is seen that silver nanoparticles appear to be the most toxic ENM with a PNEC value of 0.012 µg/L. PNEC values around 1 µg/L were obtained for CNTs, CuO and ZnO. CeO2 and TiO2 seem to be the least toxic with PNEC values of 5.2 and 18 µg/L, respectively.

Table 13 - Overview of the PNEC values derived in this report.

ENM	AF	PNEC, µg/L
TiO_2	50	18
ZnO	50	2.5
Ag	100	0.012
CNTs	50	0.84
CuO	100	0.34
nZVI	100	5
CeO_2	50	5.2
QDs	-	-
CB	-	-

- : PNEC estimation not possible due to lack of effect data.

6. Discussion

6.1 PNEC estimates and comparison with literature values

In Chapter 5 ecotoxicological studies for each of the ENMs have been reviewed, assessed for their risk assessment adequacy and PNEC estimations have been derived based on the most adequate study for regulatory use. For the sake of comparison, Table 14 below presents an overview of PNEC values for the selected ENMs revealed in this report, both the PNEC values derived using the data selection approach developed in this report (see Chapter 5), the PNEC values for the ENMs retrieved from the open literature and the PNEC values published by ECHA for both the ionic, bulk and nano form (see Chapter 3). It is also stated which PNEC approach and which AF that were used to derive the different PNEC values, but for clarity of the table the main concerns associated with the PNEC values derived in this report have been left out and readers are referred to Chapter 5 for details. In this way all worst case scenarios will be assessed, i.e. whether it is the ion, the bulk or the nano form that possess the lowest PNEC value.

Table 14 - Overview of PNEC (freshwater) values for different forms of the materials in this report. All values are in µg/L.

	ECHA			Scientific literature		
Material	**ion***	**bulk***	**nano***	**AF approach§**	**PSSD approach§**	**This report#**
TiO2	-	238	-	1-5.8	61	18
ZnO	20.6	20.6	-	0.042-2,194	9.9	2.5
Ag	0.04	0.04	-	0.0007-1	0.01	0.012
CNTs	-	-	430/780	40	60	0.84
CuO	7.8	7.8	-	-	0.48	0.34
nZVI	-	-	-	-	-	5
CeO_2	-	-	-	52-108	2	5.2
QDs	-	-	-	-	-	-
CB	-	-	5,000/50,000	-	-	-

-: indicates that no data was identified or that PNEC values could not be established at present;

*: For "ion" and "bulk" the SSD approach with AFs of 1-3 were used, except for bulk TiO_2 where the AF approach was used applying an AF of 100, for "nano" the AF approach was used applying an AF of 10 for CNTs and 1,000 and 100 for CB, respectively, see Chapter 3 for further details and references;

§: AF: AF approach applying an AF of 1,000 except for CeO_2, where an AF of 50 was applied and PSSD: PSSD approach where varying AF are used to derive NOEC values from EC_X values, see Chapter 3 for further details and references;

#: PNEC values based on the AF approach applying an AF of 50 (except for Ag, CuO and nZVI, where 100 was used), see Chapter 5 for further details and references. It must be noted that the PNEC estimations performed in Chapter 5 are based on the assumptions that 1) the current test methods are applicable to nanomaterials, and 2) that the current extrapolation methods are valid for nanomaterials.

By using the data selection approach for ENMs, as presented in Chapter 4, the range of studies considered adequate for regulatory use were selected and PNEC values were obtained in the range from 12 ng/L to 18 µg/L for the ENMs used as case materials in this study (see Table 14). Hence, the PNEC values span several orders of magnitudes, depending on the ENM. This is not surprising as the materials are very different in chemical composition. It should be noted that no pattern in

organism (algae or daphnia) sensitivity was seen across materials in the chronic studies included for PNEC estimations.

The metal-containing ENMs may be grouped with respect to the order of magnitude of the estimated PNEC values: For titanium and cerium dioxide ENMs the PNEC values were in the order of 10 µg/L, the copper and zinc oxide ENMs around 1 µg/L, and Ag ENM as the most toxic at a level of a few ng/L. Furthermore, the PNEC estimated for CNTs was in the order of 1 µg/L. It is important to note that CNTs are truly novel materials that exist only in a nano form and for which no comparable larger (or smaller) sizes analogue exists.

Figure 12 provides a visual comparison between the PNEC values for the different forms of the materials selected as case materials in this report. When comparing the values listed in Table 14 for the bulk (ECHA), ionic (ECHA), and nano (this report) forms of the ENMs it is seen that the PNEC values for CuO-NPs is around 23 times lower than the corresponding PNEC for the ion and bulk forms of Cu. For ZnO-NPs and Ag-NPs the PNEC values are nine and three times lower than for the ion and bulk forms, respectively. To what extent this reflects the ion dissolution, dose metric or exposure quantification is not possible to verify at the current time and knowledge base. However, given the relatively low AF of 50 used for the PNEC estimations in this report, it does not seem to be the AF that is causing these lower PNEC values for ENMs compared to other forms of the materials.

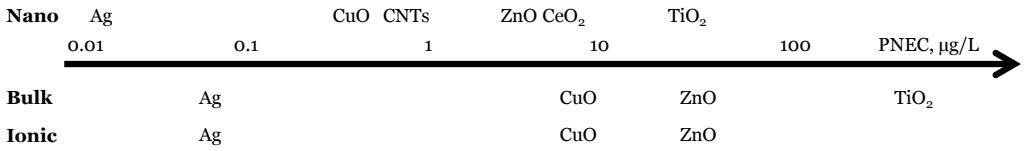


Figure 12 –Graphical presentation on a logarithmic scale of the $PNEC_{freshwater}$ values derived in this report (Nano) compared with the PNEC values for the bulk and ionic forms retrieved from ECHA.

PNEC values found in the open literature for the nano form are derived using two different methods. Comparing the PNEC values found in this report with the PNEC values derived using the PSSD approach by Gottschalk and co-workers (Gottschalk, Kost et al. 2013, Jacobs, Gottschalk et al. In prep) there is a good agreement, despite the fundamental different nature of the two estimation methods (see Chapter 3 for details). However, for CNTs the PSSD value is two orders of magnitude higher than the value derived using the AF approach, whereas the PNEC values for all other ENMs are in the same order of magnitude. It has to be noticed, that the PSSD derived PNEC values for CuO and CeO_2 were based on a more focused data set, whereas the data for the other ENMs were from a broader data set. The strength of the PSSD approach is the use of as many existing effect data as possible, however, it is at the same time a drawback as outliers and/or large variation in the dataset may get large influence on the PNEC estimation in cases where only few effect data are available. Since the variation in data is not necessarily due to differences in organism sensitivity, but may stem from lack of control of the actual exposure during testing (see Chapter 4 and 6.2), the outcome of the PSSD modelling must be interpreted with caution.

Comparing with the PNEC values found in literature estimated using the AF approach, the picture is less clear. For Ag and ZnO the AF PNEC values found in the literature span several orders of magnitude. For TiO_2, CNTs and CeO_2 the variation is much less. This reflects the availability of PNEC values found in the literature, were only few studies have published PNEC values for the last mentioned ENMs, but a range of studies have published PNEC values for the first mentioned ENMS. Not surprisingly, for Ag and ZnO the PNEC values derived in this report fit well within the large interval published in the literature. For TiO_2 the PNEC values are within the same order of magnitude, but for CNTs and CeO_2 the PNEC values derived in this report are about 1-2 orders of magnitude lower than found in the literature.

While it is tempting to conclude that the lower PNEC values found for ENMs in this report compared to ECHA registration is due to nano-specific effects, this is not supported by the findings of the studies used to estimate the PNECs. Only in a few studies nano-specific effects were included and found to be more sensitive endpoints compared to traditional endpoints for ecotoxicity tests.

In this report there has been a strong emphasis on data selection prior to PNEC estimation. The approach developed and presented in Chapter 4 thus allows for inclusion of non-standardized tests and endpoints provided that the relevance and reliability is sufficiently high taking a range of nano-specific test concerns into account. This procedure provided a more transparent, criteria based, selection of studies adequate for PNEC estimation and may have contributed significantly to the lower PNEC values found in this report compared to ECHA registrations. Similar for the PNEC values found in the literature, the PNEC values derived in this report most likely contribute to narrowing the large interval for Ag and ZnO and substantiating the level for TiO_2, CNTs and CeO_2. Finally, it should be emphasized that this report have established PNEC values for the nano forms of CuO and nZVI, values which could neither be found in the open literature nor in ECHA registrations.

6.2 Validity of current approaches for PNEC estimation to NMs

The current paradigm for PNEC derivation (using either the AF or the SSD approaches, as described in Chapter 2), is that in principle these are suitable for evaluation of ENMs. Both approaches were also considered to be applicable to ENMs by the RIPoN3 project (Aitken, Bassan et al. 2011). Hence there is at present no nano-specific arguments included in the estimation methods for PNEC estimation that would change the way assessment factors are selected or the way SSD estimations are to be carried out.

It is, however important to note that the validity of the fundamental assumption, i.e. that PNECs for ENMs can be estimated as though they were dissolved chemicals, has not been addressed. Given the range of nano-specific concerns listed in the data selection criteria presented in Chapter 4 and elaborated on below in sections 6.2.1-6.2.3 below, it is at present not possible to claim that the use of the current approaches ensure that organisms will be protected at concentrations below the derived PNEC. In other words, specific circumstances related to ENMs, which differ from conventional chemicals, could likely affect the validity of the approach for deriving PNEC in an unpredictable manner (Baun, Hartmann et al. 2009).

6.2.1 Nanomaterial and experimental issues

The PNEC estimations are traditionally based on laboratory studies carried out under standardized conditions with well-defined exposure conditions. However, the inherent properties of ENMs present a range of challenges to this notion when toxicity tests are carried out. First of all, there are currently no internationally established guidelines for proper test performance when ENMs are studied in ecotoxicity tests. This holds true for the actual toxicity testing as well as for the crucial handling of ENMs prior to testing. Thus, procedures for preparing stable suspensions of the ENMs in a reproducible manner as well as for analysing the actual exposure (e.g. the size distributions, state of agglomeration, and number of particles) before, during and after testing are not fully developed (or even lacking). It is therefore in many cases not possible to verify if the observed effect is a function of the applied nominal concentration, size, or number of particles. A testing strategy that follows more or less blindly the established standard test set-ups for traditional chemicals to ENMs potentially, results in questionable outcomes with respect to the reliability of the test performance due to the interactions that the inherent properties of the ENMs may have under the currently defined testing conditions.

A number of transformation processes during testing may furthermore influence the effective concentrations experience by the test organisms. Metal ENMs may undergo some degree of dissolution, i.e. a certain fraction of the particulate state will go into solution and thereby alter the properties (and concentrations) of the tested material (Hartmann, Skjolding et al. 2014). The ENMs may furthermore undergo some degree of agglomeration, which is dependent on several factors like ionic strength of the testing medium, choice of medium constituents, time, presence of test organism (Hartmann, Skjolding et al. 2014). These transformations have furthermore been found to be concentration dependent (Hartmann, Skjolding et al. 2014). These processes do also take place in the natural environment, but may hamper interpretation of test results and hence data reliability as the bioavailability of the tested material may be altered in a concentration- and time-dependent manner induced by the testing conditions chosen for the supposedly controlled laboratory studies (Sørensen, Baun In Press). It may be tempting to call for increasing the environmental realism of standard tests, however this will most likely be at the expense of reproducibility and comparability violating the fundamental prerequisite of constant exposure during standard exotoxicity testing.

These physico-chemical challenges mentioned above are not unique for ENMs, as e.g. dissolution issues are well known for highly lipophilic or sparingly soluble chemicals, but the situation is further complicated by a gradual transformation of the ENM-state (Sørensen, Baun In Press). On the other hand, these phenomena are also encountered for "difficult substances" like fast degrading or very lipophilic compounds. Rosenkrantz (2013) compared with degradable chemicals, where a steady exposure concentration can be difficult to maintain throughout the exposure duration. Parallels for ENMs may therefore be drawn to traditional chemicals and especially the "difficult substances" for which specific guidelines for ecotoxicity testing exist (OECD 2000), but the analytical chemical methods and instrumentations to accurately quantify the exposure concentrations before, during, and after testing remains a major challenge when performing ecotoxicity tests of ENMs.

There are potentially lessons to be learned from the risk assessment of metals, sparingly soluble chemicals and pharmaceuticals that can be transferred to ENMs. For example it is known for metals that their speciation is dependent on e.g. the water hardness. The environmental quality standards (EQSs) have for some metals have therefore been set taking water hardness into account (EU 2008). Similarly, in risk assessment of strongly sorbing chemicals, their sorption coefficients may be taking into account when calculating EQSs. pH is also well known to have an influence on the speciation of metals as well as of organic acids and bases, where only the neutral fraction of the compound is assumed to contribute to the overall toxicity (Rosenkrantz 2013, Trapp, Franco et al. 2010). Such approaches could be transferred to ENMs where speciation can be 'translated' into an aggregation/agglomeration behaviour, which can influence their toxicity.

Furthermore, the dose metric applied represents a challenge to the current procedures for PNEC estimation. In order to derive a relevant and reliable PNEC value for risk assessment, the underlying ecotoxicological study needs to consider an appropriate dose metric for the studied ENM. As mentioned in the Best Practice for REACH nano registrants (ECHA 2014) a single measure of mass is not adequate for quantifying the exposure of ENMs. This requires further studies of how to sufficiently quantify the exposure in ecotoxicological studies. Particle number or specific surface area have been suggested as more appropriate dose-metrics (Van Hoecke, Quik et al. 2009, Van Hoecke, De Schamphelaere et al. 2008, Arvidsson, Molander et al. 2011), but the number of studies that have applied these metrics is so far much to few to draw any conclusions on whether these novel metrics represent better alternatives to expressing the effective concentrations compared to the traditional mass-based concentrations.

When comparing the reservations and gaps associated with the PNEC estimations for each ENM in Chapter 5 with the description of ENM fate and behaviour in water in (Hartmann, Skjolding et al. 2014), it is possible to divide some of the ENMs in groups for which it is more or less the same

concerns that are related to different materials:

- For all ENMs aggregation and agglomeration is of very high importance, but especially for TiO_2 and CeO_2 it is in practice difficult to maintain these particles in stable suspension. Sedimentation of TiO_2 and CeO_2 ENMs are often reported and there is a risk of biological endpoint being influenced by this behaviour. This may lead to physical effects on test organisms, e.g. entrapment of daphnids in larger agglomerates causing immobilization.

- For the Ag, ZnO, and CuO ENMs the possible dissolution in the test medium and release of ionic metal is often used to explain the found toxicity. The dissolved metal ion is most often found to be more toxic than the corresponding ENM, though exceptions have been found. It is not trivial to quantify this dissolution under actual test conditions since the dissolution depends on ENM properties like size, surface area and coating, as well as on media composition (e.g. ionic strength and pH). Furthermore, the dissolution is time-dependent and thus on-going from preparation of stock suspensions prior to testing and throughout the test duration (Sørensen, Baun In Press).

6.2.2 Biological issues

With regard to the biological effect monitored and used for determination of PNEC, there is always the question of whether the appropriate organisms and endpoints were studied. The traditional approaches work well when it comes to narcotic acting chemicals, but has been shown to fail when reactive or specifically acting chemicals like pesticides and pharmaceuticals are tested (Agerstrand, Kuester et al. 2011). ENMs may have specific effects differing from traditional chemicals and it is at present unknown whether the traditionally used test organisms are sensitive towards these effects. Therefore other types of organisms, than the organisms in the traditional base-set, may be more appropriate to study in a risk assessment context. However, the validity of such "novel" effects and alternative endpoints in a regulatory context can at present not be evaluated and a continued scrutiny of the open scientific literature is needed to make sure that the most sensitive organisms are included in future PNEC estimations.

A further complication posed by ENMs is that they themselves may influence the analytical methods used for quantifying effects. This has been shown for instance for estimations of growth rates of algae in which the quantification methods were influenced by the presence of ENMs (Hartmann, Skjolding et al. 2014) and with regards to physical effect due to high concentrations applied in standard tests. This may give rise to biological effects encountered in laboratory studies that will not occur at lower more environmentally realistic concentrations in tests or in nature (Baun, Hartmann et al. 2009).

6.2.3 Risk assessment issues

The current use of extrapolation approaches from results obtained in laboratory studies to protective concentrations in the environment relies on the basic toxicological notion that a higher concentration will lead to higher effects. Or in other words: In standardized tests the effects monitored should only be a function of the concentration since all other influencing factors are held constant throughout the duration of the experiment. Furthermore, a standardized aquatic toxicity test has a defined applicability range, as stated e.g. in the ISO 6341 test for immobilization of Daphnia magna: "This method is applicable to: chemical substances which are soluble under the conditions of the test, or can be maintained as a stable suspension or dispersion under the conditions of the test" (ISO 2012). Thus, monotonous concentration-response curves and stable suspensions during testing are required for the ecotoxicity data to be valid for risk assessment purposes. When ENMs are tested in standard ecotoxicity tests these prerequisites are challenged due to the fact that the particles will often aggregate or agglomerate as a consequence of the testing media used, but also due to the increasing concentrations needed to establish concentration-

response curves. Thus, a given concentration of small particles may have become larger particles during incubation and under testing conditions. The overall concentration remains the same, but the bioavailability of the particles may decrease and no effects will be observed at the end of the test duration. This should be considered as an artefact created by the test design, and if the density of particles were less, an effect may have been observed. For traditional chemicals comparable issues like volatilisation, sorption and degradation have been sought solved by using e.g. closed bottles, passive dosing or flow-through systems. However, similar techniques have not yet evolved for ENMs. Furthermore, for soluble chemicals it is possible to quantify the volatilisation and other loss processes and take this into account when expressing the test result. This is not possible to the same extend for the transformation processes that ENMs undergo during testing. The links between transformed states of the ENMs (e.g. dissolved or agglomerate forms) and biological effects are at present unknown. But it is known that transformation influence the test results. For PNEC estimation by application of an AF this constitutes a major problem for the validity of the extrapolation from standardized tests, since environmental effects may occur at lower concentrations than those used in the standardized tests. Thus, it is questionable whether the PNEC established by application of an AF will indeed be protective.

6.3 Implications of the identified gaps for ENM Safety Assessment

From the identified literature studies listed in Table 6 in Chapter 3 and the gap analysis carried out for each ENM in Chapter 5 a number of general gaps with respect to establishment of PNEC values for nanomaterials can be identified:

1. *Limited number of studies at different trophic levels:* It is generally found that the diversity of organisms tested is very low with respect to the trophic level. This had the implication that for all of the ENMs only the application factor approach could be used for PNEC estimation. While SSD determination may indeed be a preferred option for ENMs a significant amount of additional studies are needed for all materials in order for the SSD to provide valid estimates. The alternative approaches, e.g. estimation of PNEC by PSSD remains to be validated.

2. *Lack of studies from other environmental compartments than the aqueous compartment:* For all of the ENMs included in this report there is a pronounced lack of studies from other environmental compartments than freshwater. This lack prevented PNEC estimations for other environmental compartments and this constitutes a serious lack of data for further CSA in the context of REACH. Here PNEC-values are required for e.g. wastewater organisms, marine water, soils and sediments. For conventional chemicals there is an option of extrapolating from freshwater tests to other compartments by the use of partitioning coefficients. However, the behaviour of ENMs in complex matrices like sludge, soils and sediments is not well-understood at present and the use of partitioning coefficients for interpolation between compartments is not valid. Thus, this data gap can only be closed by additional testing in the compartments at question.

3. *Most studies focused on acute toxicity:* The literature review of the nine ENMs selected as case materials in this report shows that the vast majority of currently available ecotoxicity data stem from short-term acute tests. These are required for classification and labelling purposes and also to establish the base level for PNEC estimation. For the ENMs for which it was possible to establish a PNEC value, results from chronic tests were available from two different trophic levels only. In the AF approach this leads to a lowering of the AF from 1,000 to 50. However, typically no more than two long-term studies were available and it may be questioned whether the uncertainty in the extrapolation from laboratory tests to environmental protection values has decreased sufficiently to justify the lower AF. To

obtain less uncertainty in the determination of PNEC in CSA more studies focussed at chronic endpoints are needed.

4. *Most studies focused on zooplankton:* It was also shown, that overall the majority of effect studies focused on testing daphnia (50%) in comparison to algae (20%) and fish (30%). Acute studies were conducted on all three trophic levels, but hardly any chronic fish studies were performed. The reason for this is possibly the high expenses associated with longer term fish studies as well as other ethical standards concerning fish studies. For algae obvious technical challenges exist in the sense of quantifying growth when using counting techniques or shading of the light from the particles. In addition to this, daphnia may be a target organism due to the filter feeding nature of this trophic level.

5. *Testing of high ENM concentrations:* As explained in detail in section 6.2.1 the often high concentration and environmentally unrealistic concentrations used in standardized ecotoxicity tests may influence the ENM behaviour and bioavailability in the test medium. This may lead to false-negative results. Furthermore, at high particle concentrations physical effects like organism entrapment leading to loss of mobility or shading leading to reduced growth of algae may take place. These effects are not likely to occur at the lower concentrations and should therefore be regarded as false-positive effects that are not valid for PNEC estimation.

As highlighted by Som, Nowack et al. (2013) the "quality of published data is crucial for the process of risk assessment". This is true for both conventional and alternative approaches to PNEC estimation and risk characterisation. A general and underlying assumption is that solid and valid effect data must be used for deriving proper PNEC values. As shown through the literature review in this report there are challenges and obvious problems regarding the current framework for deriving PNEC values: 1) that effect studies are based on guidelines developed for soluble chemicals and therefore not suitable for nanomaterials and 2) that effect studies are assessed for their risk assessment adequacy according to the Klimisch score, which by nature favours studies conducted according to GLP and in accordance with (current) guidelines.

Our specific recommendation for PNEC estimations of nanomaterials therefore involves a transparent and consistent assessment of the adequacy of the given effect study for the purpose of risk assessment. Such an assessment framework has therefore been developed and is described in details in Chapter 4.

The results of applying the developed framework are consistently and transparently assessed effect studies. The result contains both an assessment of the relevance as well as the reliability, seen in a nanomaterial perspective, of the study. This enables the selection of the most solid effect study suitable for risk assessment, i.e. PNEC estimation.

Regarding the estimation of PNEC values for nanomaterials the major gap is the lack of underlying data adequate for risk assessment purposes. There is a general lack of reliable data, in the sense that despite a wide range of data have been performed according to accepted international guidelines (or modification thereof), they cannot be fully trusted to yield accurate and conservative estimates of the toxicity of an ENM. This is highly influenced by varying exposure conditions during the ecotoxicological testing, which constitute a violation of the underlying assumption of constant exposure concentration during testing. Constant exposure concentrations, eventually leading to constant organism concentrations and further to constant target location/organ concentrations, are prerequisites for valid effect data (Mackay, McCarty et al. 2014).

It should be noted, that the applied assessment factors, cannot correct for improperly conducted effect studies, or effect studies conducted according to guidelines not suited for nanomaterials. As

long as the effects studies are not sufficiently reliable, this will have implications on the derived PNEC value and must be taken into consideration in the subsequent risk assessment. There is thus an urgent need for proper guidelines for ecotoxicological studies for nanomaterials, both regarding the toxic mechanism as well as the influence of physico-chemical properties on the exposure conditions.

7. Conclusion

This report provides data on environmental effects of nine selected engineered nanomaterials (ENMs) in the form of Predicted No-effect Concentration (PNEC) values. In the current regulatory framework for safety assessment of chemicals and nanomaterials in EU it is assumed that PNEC derivation using either the assessment factor (AF) or species sensitivity distribution approaches are valid for deriving PNEC values for ENMs as well. Therefore, these approaches were applied to the nine selected ENMs in this report.

A literature review did however reveal three alternative methods for PNEC estimation of ENMs. One method providing indicative PNEC values establishing the order of magnitude of PNEC, another is based on PNEC for dissolved metal ion applying an additional assessment factor of two and the third used a probability modelling of a modified species sensitivity distribution applying a range of AFs to obtain NOEC values. These alternative methods should be compared to the traditional approaches in future studies, however this was not the aim of the present report.

The current approach to data selection for PNEC estimation was found to favour studies performed according to GLP and following established guidelines developed for soluble chemicals. As non-GLP and non-guideline studies tailor-made for effect studies of ENMs might give more reliable and relevant results, a transparent risk assessment adequacy evaluation framework was developed focusing on the reliability of the study in respect of ENM characterisation, quantification and study set-up. This framework ensured the use of regulatory adequate effect studies for PNEC derivation.

It was found that the diversity in tested organisms as well as trophic levels was low. Furthermore, hardly any chronic studies on fish were found. This has the consequence that it was not possible to perform SSD modelling, and thus PNECs were estimated solely using the AF approach.

Most studies, acute as well as chronic, focused on effect towards freshwater crustaceans (especially daphnia organisms), but in many cases also chronic studies on algae were found. For most of the selected nanomaterials this resulted in the use of an AF of 50, except for Ag, CuO where an acute study revealed a lower effect concentration resulting in the use of an AF of 100. In the acute studies daphnia and algae were identified as the most sensitive of the tested organisms. If chronic fish studies were available, this would lower the AF, but most likely only have low or no influence on the PNEC value.

There is a pronounced lack of studies from other environmental compartments than freshwater. As it is was found not to be scientifically valid to interpolate among compartments, PNEC for other compartments than freshwater can only be established by additional testing in the compartments in question. This includes tests with wastewater bacteria, soil organisms, pelagic marine organisms, as well as sediment-living organisms in fresh and salt water.

The PNEC values presented in this report are in accordance with the present state-of-knowledge but are based on the assumptions that 1) the current test methods are applicable to nanomaterials, and 2) that the current extrapolation methods are valid for nanomaterials. These assumptions remain to be validated, especially when high concentrations are tested, as these may influence the ENM behaviour and bioavailablility, probably resulting in false-negative results. The applied AFs are not designed to cover uncertainties in the quantification of the exposure level (of ENMs). The values

reported here should therefore be taken as indicative for the order of magnitude for the PNEC given the current regulatory recommendations for PNEC estimation and not be used as the definitive protective concentration for the environment.

Besides establishing a wider range of (chronic) effect data, the major way to improve the validity and magnitude of the estimated PNEC values would be to apply and/or develop techniques for improved exposure conditions and confident exposure quantifications. Whether such improvements will result in lower or higher PNEC values, is beyond the scope of this report to assess.

By using the data selection approach for ENMs developed in this report, the studies adequate for regulatory use were selected and PNEC values were derived. Ag was found to be the most toxic with a PNEC value of 12 ng/L and TiO_2 was found to be the least toxic with a PNEC value 18 µg/L for the ENMs used as case materials in this report. The PNEC values for CNTs, CuO, ZnO and CeO_2 were found in between. The PNEC value for nZVI (5 µg/L) cannot be compared with the rest, as it was derived as an intermittent PNEC due to the short-lived nature of nZVI under realistic environmental conditions. It was furthermore found that it is at present not possible to establish PNEC values for CB and QDs. See Table 14 from the Discussion, Chapter 6 above, and reprinted below.

Table 14 - Overview of PNEC (freshwater) values for different forms of the nanomaterials in this report. All values are in µg/L.

	ECHA			Scientific literature		
Material	**ion***	**bulk***	**nano***	**AF approach§**	**PSSD approach§**	**This report#**
TiO2	-	238	-	1-5.8	61	18
ZnO	20.6	20.6	-	0.042-2,194	9.9	2.5
Ag	0.04	0.04	-	0.0007-1	0.01	0.012
CNTs	-	-	430/780	40	60	0.84
CuO	7.8	7.8	-	-	0.48	0.34
nZVI	-	-	-	-	-	5
CeO_2	-	-	-	52-108	2	5.2
QDs	-	-	-	-	-	-
CB	-	-	5,000/50,000	-	-	-

-: indicates that no data was identified or that PNEC values could not be established at present;

*: For "ion" and "bulk" the SSD approach with AFs of 1-3 were used, except for bulk TiO_2 where the AF approach was used applying an AF of 100, for "nano" the AF approach was used applying an AF of 10 for CNTs and 1,000 and 100 for CB, respectively, see Chapter 3 for further details and references;

§: AF: AF approach applying an AF of 1,000 except for CeO_2, where an AF of 50 was applied and PSSD: PSSD approach where varying AF are used to derive NOEC values from EC_X values, see Chapter 3 for further details and references;

#: PNEC values based on the AF approach applying an AF of 50 (except for Ag, CuO and nZVI, where 100 was used), see Chapter 5 for further details and references. It must be noted that the PNEC estimations performed in Chapter 5 are based on the assumptions that 1) the current test methods are applicable to nanomaterials, and 2) that the current extrapolation methods are valid for nanomaterials.

8. References

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Appendices

Table 15 – Detailed overview of the selected studies on environmental effects of titanium dioxide (TiO_2) nanoparticles. Reliability and relevance were assessed according to the article by Hartmann, Lützhøft et al. (In prep) and the associated spreadsheet developed for the scoring of different criteria used to assess the reliability and relevance.

Trophic level	Organism	Acute/chronic	Duration, h	Endpoint	Quantification of endpoint	Value, mg/L	Reliability	Relevance	Reference	Comment
Daphnia	*D. magna*	Acute	48	Immobilisation	EC50	>100	nRi2	Re1	(Wiench, Wohlleben et al. 2009)	M4-medium
Daphnia	*D. magna*	Acute	48	Immobilisation	EC50	>100	nRi2	Re1	(Wiench, Wohlleben et al. 2009)	spring water
Daphnia	*D. magna*	Acute	48	Immobilisation	EC10	>100	nRi2	Re1	(Wiench, Wohlleben et al. 2009)	M4-medium
Daphnia	*D. magna*	Acute	48	Immobilisation	EC10	3.7	nRi2	Re1	(Wiench, Wohlleben et al. 2009)	spring water
Daphnia	*D. magna*	Chronic	504	Mortality	EC10	31.5	nRi2	Re1	(Wiench, Wohlleben et al. 2009)	M4-medium
Daphnia	*D. magna*	Chronic	504	Mortality	EC50	66.1	nRi2	Re1	(Wiench, Wohlleben et al. 2009)	M4-medium
Daphnia	*D. magna*	Chronic	504	Cumulative offspring	EC10	5.02	nRi2	Re1	(Wiench, Wohlleben et al. 2009)	M4-medium
Daphnia	*D. magna*	Chronic	504	Cumulative offspring	EC50	26.6	nRi2	Re1	(Wiench, Wohlleben et al. 2009)	M4-medium
Bacteria	*V. fischeri*	Acute	0,5	Luminiscence	NOEC	>20000	nRi3	Re1	(Heinlaan, Ivask et al. 2008)	
Bacteria	*V. fischeri*	Acute	0,5	Luminiscence	EC50	>20000	nRi3	Re1	(Heinlaan, Ivask et al. 2008)	
Daphnia	*D. magna*	Acute	48	Mortality	EC50	>20000	nRi3	Re1	(Heinlaan, Ivask et al. 2008)	
Daphnia	*T. platyurus*	Acute	24	Mortality	NOEC	>20000	nRi3	Re1	(Heinlaan, Ivask et al. 2008)	
Daphnia	*T. platyurus*	Acute	24	Mortality	EC50	>20000	nRi3	Re1	(Heinlaan, Ivask et al. 2008)	
Algae	*P. subcapitata*	Chronic	72	Growth	EC10	3.3	nRi2	Re1	(Hartmann, Von der Kammer et al. 2010)	67% anatase; 33% amorphous
Algae	*P. subcapitata*	Chronic	72	Growth	EC50	241	nRi2	Re1	(Hartmann, Von der Kammer et al. 2010)	
Algae	*P. subcapitata*	Chronic	72	Growth	NOAEC	<0.5	nRi2	Re1	(Lee, An 2013)	21 nm, 73% anatase, 18% rutile, 9% amorphous
Algae	*P. subcapitata*	Chronic	72	Growth	EC50	2,53	nRi2	Re1	(Lee, An 2013)	21 nm, 73% anatase, 18% rutile, 9% amorphous
Algae	*Chlorella*	Chronic	72	Growth	NOEC	0.89	nRi2	Re1	(Sadiq, Dalai et al. 2011)	<25 nm oxide anatase
Algae	*Chlorella*	Chronic	72	Growth	EC50	16.12	nRi2	Re1	(Sadiq, Dalai et al. 2011)	<25 nm oxide anatase
Algae	*Scenedesmus*	Chronic	72	Growth	NOEC	1.2	nRi2	Re1	(Sadiq, Dalai et al. 2011)	<25 nm oxide anatase
Algae	*Scenedesmus*	Chronic	72	Growth	EC50	21.2	nRi2	Re1	(Sadiq, Dalai et al. 2011)	<25 nm oxide anatase
Algae	*P. subcapitata*	Chronic	72	Growth	NOEC	0.984	nRi3	Re1	(Aruoja, Dubourguier et al. 2009)	
Algae	*P. subcapitata*	Chronic	72	Growth	EC50	5.83	nRi3	Re1	(Aruoja, Dubourguier et al. 2009)	
Daphnia	*D. magna*	Acute	48	Survival	LC50	7.75	nRi3	Re1	(Das, Xenopoulos et al. 2013)	
Daphnia	*D. magna*	Chronic	576	Mortality	EC20[a]	4.5[a]	nRi3	Re1	(Das, Xenopoulos et al. 2013)	

Daphnia	*D. magna*	Chronic	576	Offspring reduction	EC50[a]	4.5[a]	nRi3	Re1	(Das, Xenopoulos et al. 2013)	
Daphnia	*D. magna*	Chronic	576	Days to brood	Significant	4.5	nRi3	Re1	(Das, Xenopoulos et al. 2013)	
Daphnia neonates	*C. dubia*	Acute	48	Death	LC50	>10	nRi2	Re2	(Griffitt, Luo et al. 2008)	
Daphnia adults	*D. pulex*	Acute	48	Death	LC50	>10	nRi2	Re2	(Griffitt, Luo et al. 2008)	
Fish juveniles	*D. rerio*	Acute	48	Death	LC50	>10	nRi2	Re2	(Griffitt, Luo et al. 2008)	
Fish adults	*D. rerio*	Acute	48	Death	LC50	>10	nRi2	Re2	(Griffitt, Luo et al. 2008)	
Algae	*C. reinhardtii*	Chronic	72	Growth inhibition	EC50	10	nRi2	Re2	(Wang, Zhang et al. 2008)	Freshwater, based on the NP
Algae	*C. reinhardtii*	Acute	3	Stress response genes	Significant	1	nRi2	Re2	(Wang, Zhang et al. 2008)	Freshwater, based on the NP
Algae	*C. reinhardtii*	Acute	3	Lipid peroxidation	Significant	1	nRi2	Re2	(Wang, Zhang et al. 2008)	Freshwater, based on the NP
Bacteria	*E. coli*	Acute	Ns	β-galactosidase	EC50	Not toxic	nRi2	Re2	(Pokhrel, Silva et al. 2012)	Soil
Fish embryos	*D. rerio*	Acute	120	Survival	LC50	84	nRi2	Re2	(Vicario-Pares, Castanaga et al. 2014)	100% rutile, 60 nm
Fish embryos	*D. rerio*	Acute	120	Survival	LC50	>100	nRi2	Re2	(Vicario-Pares, Castanaga et al. 2014)	55% rutile, 45% anatase, <100 nm
Fish embryos	*D. rerio*	Acute	120	Survival	LC50	>100	nRi2	Re2	(Vicario-Pares, Castanaga et al. 2014)	10% rutile, 70% anatase, 10-20 nm
Protozoa	*P. multimicronucleatum*	Acute	48	Mortality	LC50	7,215	nRi2	Re2	(Li, Chen et al. 2012)	5.1 nm
Amphibian	*X. laevis*	Chronic	96	Malformations	Significant	10	nRi2	Re2	(Bacchetta, Santo et al. 2012)	<100 nm
Fish embryos	*D. rerio*	Acute	72	Mortality	LC50	>2,000	nRi2	Re1	(Felix, Ortega et al. 2013)	Anionic polyelectrolyte coating

a: estimated from graphs

Table 16 – Detailed overview of the selected studies on environmental effects of zinc oxide (ZnO) nanoparticles. Reliability and relevance were assessed according to the article by Hartmann, Lützhøft et al. (In prep) and the associated spreadsheet developed for the scoring of different criteria used to assess the reliability and relevance.

Trophic level	Organism	Acute/chronic	Duration, h	Endpoint	Quantification of endpoint	Value, mg/L	Reliability	Relevance	Reference	Comment
Bacteria	*E. coli*	Acute	Ns	β-galactosidase	EC_{50}	80	nRi2	Re2	(Pokhrel, Silva et al. 2012)	Soil, octacecylamine coating
Daphnia	*D. magna*	Acute	48	Immobilisation	EC50, M4-medium	7,5	nRi2	Re1	(Wiench, Wohlleben et al. 2009)	Uncoated
Daphnia	*D. magna*	Acute	48	Immobilisation	EC10, M4-medium	5,2	nRi2	Re1	(Wiench, Wohlleben et al. 2009)	Uncoated
Daphnia	*D. magna*	Acute	48	Immobilisation	EC50, M4-medium	1,1	nRi2	Re1	(Wiench, Wohlleben et al. 2009)	HP1 coating
Daphnia	*D. magna*	Acute	48	Immobilisation	EC10, M4-medium	0,2	nRi2	Re1	(Wiench, Wohlleben et al. 2009)	HP1 coating
Daphnia	*D. magna*	Acute	48	Immobilisation	EC50, spring water	>100	nRi2	Re1	(Wiench, Wohlleben et al. 2009)	HP1 coating
Daphnia	*D. magna*	Acute	48	Immobilisation	EC10, spring water	2,7	nRi2	Re1	(Wiench, Wohlleben et al. 2009)	HP1 coating
Daphnia	*D. magna*	Acute	48	Immobilisation	EC50, pond water	13,4	nRi2	Re1	(Wiench, Wohlleben et al. 2009)	HP1 coating
Daphnia	*D. magna*	Acute	48	Immobilisation	EC10, pond water	9,3	nRi2	Re1	(Wiench, Wohlleben et al. 2009)	HP1 coating
Daphnia	*D. magna*	Acute	48	Immobilisation	EC50, M4-medium	1	nRi2	Re1	(Wiench, Wohlleben et al. 2009)	MAX coating
Daphnia	*D. magna*	Acute	48	Immobilisation	EC10, M4-medium	0,7	nRi2	Re1	(Wiench, Wohlleben et al. 2009)	MAX coating
Bacteria	*V. fischeri*	Acute	0,5	Luminiscence	NOEC	0,75	nRi3	Re1	(Heinlaan, Ivask et al. 2008)	
Bacteria	*V. fischeri*	Acute	0,5	Luminiscence	EC50	1,9	nRi3	Re1	(Heinlaan, Ivask et al. 2008)	
Daphnia	*D. magna*	Acute	48	Mortality	NOEC	1,5	nRi3	Re1	(Heinlaan, Ivask et al. 2008)	
Daphnia	*D. magna*	Acute	48	Mortality	EC50	8,8	nRi3	Re1	(Heinlaan, Ivask et al. 2008)	
Daphnia	*T. platyurus*	Acute	24	Mortality	NOEC	0,05	nRi3	Re1	(Heinlaan, Ivask et al. 2008)	
Daphnia	*T. platyurus*	Acute	24	Mortality	EC50	0,24	nRi3	Re1	(Heinlaan, Ivask et al. 2008)	
Algae	*P. subcapitata*	Chronic	72	Growth	NOAEC	<0,5	nRi2	Re1	(Lee, An 2013)	<100 nm
Algae	*P. subcapitata*	Chronic	72	Growth	EC50	<0,5	nRi2	Re1	(Lee, An 2013)	<100 nm
Algae	*P. subcapitata*	Chronic	72	Growth	NOEC	0,017	nRi3	Re1	(Aruoja, Dubourguier et al. 2009)	50-70 nm
Algae	*P. subcapitata*	Chronic	72	Growth	EC50	0,042	nRi3	Re1	(Aruoja, Dubourguier et al. 2009)	50-70 nm
Algae	*Stichococcus sp.*	Chronic	120	Growth	NOEC	1,3*	nRi2	Re1	(Gladis, Eggert et al. 2010)	Terrestrial, 20-60 nm, -UV
Algae	*Stichococcus sp.*	Chronic	120	Growth	EC50	8,19*	nRi2	Re1	(Gladis, Eggert et al. 2010)	Terrestrial, 20-60 nm, -UV
Algae	*Stichococcus sp.*	Chronic	120	Growth	NOEC	0,06*	nRi2	Re1	(Gladis, Eggert et al. 2010)	Terrestrial, 20-60 nm, +UV
Algae	*Stichococcus sp.*	Chronic	120	Growth	EC50	1,17*	nRi2	Re1	(Gladis, Eggert et al. 2010)	Terrestrial, 20-60 nm, +UV
Daphnia	*D. magna*	Acute	48	Mortality	LC50	1,02	nRi2	Re1	(Lopes, Ribeiro et al. 2014)	30 nm
Daphnia	*D. magna*	Acute	24	Feeding inh	EC50	1,41	nRi2	Re1	(Lopes, Ribeiro et al. 2014)	30 nm
Daphnia	*D. magna*	Acute	4	Post exp feeding inh	EC50	1,27	nRi2	Re1	(Lopes, Ribeiro et al. 2014)	30 nm
Daphnia	*D. magna*	Chronic	504	Reproduction	NOEC	no	nRi2	Re1	(Lopes, Ribeiro et al. 2014)	30 nm

Daphnia	*D. magna*	Chronic	504	Reproduction	LOEC	0,125	nRi2	Re1	(Lopes, Ribeiro et al. 2014)	30 nm
Daphnia	*D. magna*	Chronic	504	Reproduction	EC50	0,26	nRi2	Re1	(Lopes, Ribeiro et al. 2014)	30 nm
Daphnia	*D. magna*	Acute	48	Mortality	LC50	1,1	nRi2	Re1	(Lopes, Ribeiro et al. 2014)	80-100 nm
Daphnia	*D. magna*	Acute	24	Feeding inh	EC50	2	nRi2	Re1	(Lopes, Ribeiro et al. 2014)	80-100 nm
Daphnia	*D. magna*	Acute	4	Post exp feeding inh	EC50	1,91	nRi2	Re1	(Lopes, Ribeiro et al. 2014)	80-100 nm
Daphnia	*D. magna*	Chronic	504	Reproduction	NOEC	0,125	nRi2	Re1	(Lopes, Ribeiro et al. 2014)	80-100 nm
Daphnia	*D. magna*	Chronic	504	Reproduction	LOEC	0,25	nRi2	Re1	(Lopes, Ribeiro et al. 2014)	80-100 nm
Daphnia	*D. magna*	Chronic	504	Reproduction	EC50	0,36	nRi2	Re1	(Lopes, Ribeiro et al. 2014)	80-100 nm
Fish cells	*P. lucida*	Acute	2	MTT	NOEC	5	nRi2	Re2	(Luisa Fernandez-Cruz, Lammel et al. 2013)	<100 nm
Fish cells	*P. lucida*	Acute	2	MTT	NOEC	2	nRi2	Re2	(Luisa Fernandez-Cruz, Lammel et al. 2013)	<50 nm
Fish cells	*P. lucida*	Acute	2	MTT	NOEC	5	nRi2	Re2	(Luisa Fernandez-Cruz, Lammel et al. 2013)	<100 nm
Fish cells	*P. lucida*	Acute	2	NRU	NOEC	18	nRi2	Re2	(Luisa Fernandez-Cruz, Lammel et al. 2013)	<50 nm
Fish cells	*P. lucida*	Acute	2	NRU	NOEC	7	nRi2	Re2	(Luisa Fernandez-Cruz, Lammel et al. 2013)	20-30 nm
Fish cells	*P. lucida*	Acute	2	NRU	NOEC	12	nRi2	Re2	(Luisa Fernandez-Cruz, Lammel et al. 2013)	20-30 nm
Fish cells	*P. lucida*	Acute	2	LDH	NOEC	9	nRi2	Re2	(Luisa Fernandez-Cruz, Lammel et al. 2013)	<100 nm
Fish cells	*P. lucida*	Acute	2	LDH	NOEC	7	nRi2	Re2	(Luisa Fernandez-Cruz, Lammel et al. 2013)	<50 nm
Fish cells	*P. lucida*	Acute	2	LDH	NOEC	12	nRi2	Re2	(Luisa Fernandez-Cruz, Lammel et al. 2013)	<100 nm
Fish cells	*P. lucida*	Acute	2	LUCS	NOEC	18	nRi2	Re2	(Luisa Fernandez-Cruz, Lammel et al. 2013)	<50 nm
Fish cells	*P. lucida*	Acute	2	LUCS	NOEC	16	nRi2	Re2	(Luisa Fernandez-Cruz, Lammel et al. 2013)	20-30 nm
Fish cells	*P. lucida*	Acute	2	LUCS	NOEC	25	nRi2	Re2	(Luisa Fernandez-Cruz, Lammel et al. 2013)	20-30 nm
Fish embryos	*D. rerio*	Acute	120	Mortality	LC50	>10	nRi2	Re2	(Vicario-Pares, Castanaga et al. 2014)	<100 nm
Daphnia	*D. magna*	Acute	48	Survival	EC50	1,7-9,0	nRi3	Re2	(Blinova, Ivask et al. 2010)	Natural waters, 70 nm
Daphnia	*T. platyurus*	Acute	24	Mortality	LC50	1,1-6,0	nRi3	Re2	(Blinova, Ivask et al. 2010)	Natural waters, 70 nm
Protozoa	*T. thermophilia*	Acute	24	Survival	EC50	12-27	nRi3	Re2	(Blinova, Ivask et al. 2010)	Natural waters, 70 nm
Protozoa	*T. thermophilia*	Acute	4	Membrane disruption	EC50	4,3	nRi2	Re2	(Mortimer, Kasemets et al. 2010)	50-70 nm
Protozoa	*T. thermophilia*	Acute	24	Membrane disruption	EC50	6,8	nRi2	Re2	(Mortimer, Kasemets et al. 2010)	50-70 nm
Protozoa	*T. thermophilia*	Acute	4	Cell viability	EC50	5,0	nRi2	Re2	(Mortimer, Kasemets et al. 2010)	50-70 nm
Protozoa	*T. thermophilia*	Acute	24	Cell viability	EC50	8,3	nRi2	Re2	(Mortimer, Kasemets et al. 2010)	50-70 nm
Amphibian	*X. laevis*	Chronic	96	Malformations	Significant	10	nRi2	Re2	(Bacchetta, Santo et al. 2012)	<100 nm
Bacteria	Soil organism	Acute	5-7	Growth	EC50	5208#	nRi3	Re2	(Rousk, Ackermann et al. 2012)	20 nm, mineral soil
Bacteria	Soil organism	Acute	5-7	Growth	EC50	15055#	nRi3	Re2	(Rousk, Ackermann et al. 2012)	20 nm, organic soil
Protozoa	*P. multimicronucleatum*	Acute	48	Mortality	LC50	573,8	nRi2	Re2	(Li, Chen et al. 2012)	10 nm
Daphnia	*C. affinis*	Acute	48	Mortality	LC50	0,09	nRi3	Re2	(Tomilina, Gremyachikh et al. 2011)	
Daphnia	*C. affinis*	Chronic	168	Fertility	EC50	0,054	nRi3	Re2	(Tomilina, Gremyachikh et al. 2011)	
Fish embryo	*D. rerio*	Acute	72	Mortality	LC50	1589	nRi2	Re1	(Felix, Ortega et al. 2013)	PAA coating
Algae	*T. weisflogii*	Chronic	168	Growth	NOEC	0,010	nRi3	Re2	(Jarvis, Miller et al. 2013)	Marine

Daphnia	*A. tonsa*	Chronic	168	Survival	NOEC	0,010	nRi3	Re2	(Jarvis, Miller et al. 2013)	Marine
Daphnia	*A. tonsa*	Chronic	168	Reproduction	NOEC	0,168	nRi3	Re2	(Jarvis, Miller et al. 2013)	Marine
Bacteria	*B. subtilis*	Acute	6	Growth	EC90	10	nRi3	Re2	(Adams, Lyon et al. 2006)	67 nm
Bacteria	*E. coli*	Acute	6	Growth	EC14	10	nRi3	Re2	(Adams, Lyon et al. 2006)	67 nm
Bacteria	*D. magna*	Chronic	192	Survival	EC73	0,2	nRi3	Re2	(Adams, Lyon et al. 2006)	67 nm

HP1: Triethoxycaprylylsilane; a: estimated from graphs; MAX: dimethoxydiphenylsilane/triethoxycaprylylsilane crosspolymer; *: mg/cm²; inh: inhibition; exp: exposure; no: not observed; MTT: Mitochondrial activity; NRU: Lysosomal membrane integrity; LDH: Lactate dehydrogenase activity; LUCS: DNA damage; #: mg/g; PAA: Anionic polyelectrolyte;

Table 17 – Detailed overview of the selected studies on environmental effects of silver (Ag) nanoparticles. Reliability and relevance were assessed according to the article by Hartmann, Lützhøft et al. (In prep) and the associated spreadsheet developed for the scoring of different criteria used to assess the reliability and relevance.

Trophic level	Organism	Acute/chronic	Duration, h	Endpoint	Quantification of endpoint	Value, µg/L	Reliability	Relevance	Reference	Comment
Fish	*D. rerio* embryos	Subacute	72	Abnormal notochord	Difference from control	0.010	nRi3	Re2	(Yeo, Kang 2008)	
Fish	*D. rerio* embryos	Subacute	72	Edema	Difference from control	0.010	nRi3	Re2	(Yeo, Kang 2008)	
Fish	*D. rerio* embryos	Subacute	72	Weak heartbeat	Difference from control	0.010	nRi3	Re2	(Yeo, Kang 2008)	
Fish	*D. rerio* embryos	Subacute	72	Enzyme	Difference from control	0.020	nRi3	Re2	(Yeo, Kang 2008)	
Fish	*D. rerio* embryos	Subacute	72	Hatching	Difference from control	0.010	nRi3	Re2	(Yeo, Kang 2008)	
Fish	*D. rerio* embryos	Subacute	72	Genes	Difference from control	0.010	nRi3	Re2	(Yeo, Kang 2008)	
Algae	*P. subcapitata*	Chronic	96	Growth	EC50	190	nRi2	Re2	(Griffitt, Luo et al. 2008)	
Daphnia	*C. dubia* neonates	Acute	48	Death	LC50	67	nRi2	Re2	(Griffitt, Luo et al. 2008)	
Daphnia	*D. pulex* adults	Acute	48	Death	LC50	40	nRi2	Re2	(Griffitt, Luo et al. 2008)	
Fish	*D. rerio* juveniles	Acute	48	Death	LC50	7200	nRi2	Re2	(Griffitt, Luo et al. 2008)	
Fish	*D. rerio* adults	Acute	48	Death	LC50	7070	nRi2	Re2	(Griffitt, Luo et al. 2008)	
Bacteria	Nitrifying cultures	Acute	-	Growth	EC50	140	nRi3	Re2	(Choi, Hu 2008)	
Bacteria	Nitrifying cultures	Acute	0,5	Intracellular ROS	Difference from control	100	nRi3	Re2	(Choi, Hu 2008)	
Bacteria	Nitrifying cultures	Acute	0,5	Photocatalytic ROS	Difference from control	100	nRi3	Re2	(Choi, Hu 2008)	
Fish	*D. rerio* embryos	Acute	72	Mortality	LC50[a]	≈ 30000[a]	nRi3	Re2	(Asharani, Wu et al. 2008)	
Fish	*D. rerio* embryos	Acute	72	Hatching	EC50[a]	≈ 75000[a]	nRi3	Re2	(Asharani, Wu et al. 2008)	
Fish	*D. rerio* embryos	Acute	72	Distorted yolk sac	EC60-90	50000	nRi3	Re2	(Asharani, Wu et al. 2008)	
Fish	*D. rerio* embryos	Acute	72	Pericardial edema	EC60-90	50000	nRi3	Re2	(Asharani, Wu et al. 2008)	
Fish	*D. rerio* embryos	Acute	72	Bent notochord	EC60-90	50000	nRi3	Re2	(Asharani, Wu et al. 2008)	
Fish	*D. rerio* embryos	Acute	72	Decreased heart rate	EC50[a]	≈ 50000[a]	nRi3	Re2	(Asharani, Wu et al. 2008)	
Fish	*D. rerio* embryos	Acute	72	Apoptosis	Appearance	50000	nRi3	Re2	(Asharani, Wu et al. 2008)	
Algae	*C. reinhardtii*	Acute	5	Photosynthesis	EC50	89	nRi2	Re2	(Navarro, Piccapietra et al. 2008)	
Bacteria	*V. fischeri*	Acute	0,5	Luminescence	EC10	160	nRi2	Re1	(Georgantzopoulou, Balachandran et al. 2013)	
Bacteria	*V. fischeri*	Acute	0,5	Luminescence	EC50	420	nRi2	Re1	(Georgantzopoulou, Balachandran et al. 2013)	
Algae	*D. subspicatus*	Chronic	72	Growth	EC10	10	nRi2	Re1	(Georgantzopoulou, Balachandran et al. 2013)	
Algae	*D. subspicatus*	Chronic	72	Growth	EC50	34	nRi2	Re1	(Georgantzopoulou, Balachandran et al. 2013)	
Daphnia	*D. magna*	Acute	48	Immobilisation	EC10	0.4	nRi2	Re1	(Georgantzopoulou, Balachandran et al. 2013)	
Daphnia	*D. magna*	Acute	48	Immobilisation	EC50	1.2	nRi2	Re1	(Georgantzopoulou, Balachandran et al. 2013)	
Daphnia	*D. magna*	Acute	48	Survival	LC50	2.75	nRi3	Re2	(Das, Xenopoulos et al. 2013)	
Bacteria	*E. coli*	Acute	Ns	β-galactosidase	EC_{50}	6000	nRi2	Re2	(Pokhrel, Silva et al. 2012)	Soil, citrate coating

Bacteria	*E. coli*	Acute	Ns	β-galactosidase	EC_{50}	1000	nRi2	Re2	(Pokhrel, Silva et al. 2012)	Soil, PVP coating
Daphnia	*D. magna*	Acute	96	Mortality	LC56	100	nRi2	Re1	(Gaiser, Fernandes et al. 2012)	
Daphnia	*D. magna*	Chronic	504	Mortality	LC10-30	1-50	nRi2	Re2	(Gaiser, Biswas et al. 2011)	
Daphnia	*D. magna*	Chronic	504	Mortality	LOEC/EC10	5	nRi2	Re2	(Zhao, Wang 2011)	CO_3-coated, dietary exposure
Daphnia	*D. magna*	Chronic	504	Time to brood	Not significant	5	nRi2	Re2	(Zhao, Wang 2011)	CO_3-coated, dietary exposure
Daphnia	*D. magna*	Chronic	504	Offspring	Not significant	5	nRi2	Re2	(Zhao, Wang 2011)	CO_3-coated, dietary exposure
Daphnia	*D. magna*	Chronic	504	Length	Significant	5	nRi2	Re2	(Zhao, Wang 2011)	CO_3-coated, dietary exposure
Daphnia	*D. magna*	Chronic	504	Mortality	Not significant	50	nRi2	Re2	(Zhao, Wang 2011)	CO_3-coated, dietary exposure
Daphnia	*D. magna*	Chronic	504	Time to brood	Not significant	50	nRi2	Re2	(Zhao, Wang 2011)	CO_3-coated, dietary exposure
Daphnia	*D. magna*	Chronic	504	Offspring	Significant	50	nRi2	Re2	(Zhao, Wang 2011)	CO_3-coated, dietary exposure
Daphnia	*D. magna*	Chronic	504	Length	Significant	50	nRi2	Re2	(Zhao, Wang 2011)	CO_3-coated, dietary exposure
Daphnia	*D. magna*	Chronic	504	Offspring increase	Significant	2	nRi2	Re2	(Pokhrel, Dubey 2012)	Citrate-coated

-: not mentioned; a: estimated from graphs

Table 18 – Detailed overview of the selected studies on environmental effects of carbon nanotubes (CNTs) nanoparticles. Reliability and relevance were assessed according to the article by Hartmann, Lützhøft et al. (In prep) and the associated spreadsheet developed for the scoring of different criteria used to assess the reliability and relevance.

Trophic level	Organism	Acute/chronic	Duration, h	Endpoint	Quantification of endpoint	Value, mg/L	Reliability	Relevance	Reference	Comment
Algae	*Chlorella sp.*	Chronic	96	Growth	IC50	41	nRi2	Re2	(Long, Ji et al. 2012)	<10 nm, light
Algae	*Chlorella sp.*	Chronic	96	Growth	IC50	13	nRi2	Re2	(Long, Ji et al. 2012)	20-40 nm, light
Algae	*Chlorella sp.*	Chronic	96	Growth	IC50	12	nRi2	Re2	(Long, Ji et al. 2012)	60-100 nm, light
Algae	*Chlorella sp.*	Chronic	96	Growth	IC50	63	nRi2	Re2	(Long, Ji et al. 2012)	<10 nm, dark
Algae	*Chlorella sp.*	Chronic	96	Growth	IC50	37	nRi2	Re2	(Long, Ji et al. 2012)	20-40 nm, dark
Algae	*Chlorella sp.*	Chronic	96	Growth	IC50	46	nRi2	Re2	(Long, Ji et al. 2012)	60-100 nm, dark
Algae	*C. vulgaris*	Chronic	96	Growth	EC50	1.8	nRi2	Re2	(Schwab, Bucheli et al. 2011)	SR HA as suspenser
Algae	*C. vulgaris*	Chronic	96	Growth	NOEC	0.042	nRi2	Re2	(Schwab, Bucheli et al. 2011)	SR HA as suspenser
Algae	*P. subcapitata*	Chronic	96	Growth	EC50	20	nRi2	Re2	(Schwab, Bucheli et al. 2011)	SR HA as suspenser
Algae	*P. subcapitata*	Chronic	96	Growth	NOEC	1.3	nRi2	Re2	(Schwab, Bucheli et al. 2011)	SR HA as suspenser
Daphnia	*D. magna*	Acute	96	Mortality	LC50	1.9	nRi2	Re2	(Edgington, Roberts et al. 2010)	25 nm, SR HA
Daphnia	*C. dubia*	Chronic	168	Reproduction	Significant	0.125	nRi2	Re2	(Edgington, Roberts et al. 2010)	25 nm, SR HA
Fish embryo	*D. rerio*	Acute	72	Notochord	NOEC	40	nRi3	Re2	(Asharani, Serina et al. 2008)	30-40 nm
Fish embryo	*D. rerio*	Acute	72	Mortality	NOEC	40	nRi3	Re2	(Asharani, Serina et al. 2008)	30-40 nm

SR HA: Suwannee River humic acid;

Table 19 – Detailed overview of the selected studies on environmental effects of copper oxide (CuO) nanoparticles. Reliability and relevance were assessed according to the article by Hartmann, Lützhøft et al. (In prep) and the associated spreadsheet developed for the scoring of different criteria used to assess the reliability and relevance.

Trophic level	Organism	Acute/ chronic	Duration, h	Endpoint	Quantification of endpoint	Value, mg/L	Reliability	Relevance	Reference	Comment
Daphnia	*C. dubia*	Acute	48	Survival	LC50	0.0338	nRi2	Re2	(Kennedy, Melby et al. 2013)	Nanospheres tested
Daphnia	*C. dubia*	Acute	48	Survival	LC50	0.1149	nRi2	Re2	(Kennedy, Melby et al. 2013)	Nanorods tested
Algae	*P. subcapitata*	Chronic	72	Growth	EC50	0.710	nRi3	Re2	(Aruoja, Dubourguier et al. 2009)	
Algae	*P. subcapitata*	Chronic	72	Growth	NOEC	0.421	nRi3	Re2	(Aruoja, Dubourguier et al. 2009)	
Bacteria	*V. fischeri*	Acute	0.5	Luminescence	EC50	79	nRi3	Re2	(Heinlaan, Ivask et al. 2008)	
Bacteria	*V. fischeri*	Acute	0.5	Luminescence	NOEC	16	nRi3	Re2	(Heinlaan, Ivask et al. 2008)	
Daphnia	*D. magna*	Acute	48	Mortality	LC50	3.2	nRi3	Re2	(Heinlaan, Ivask et al. 2008)	
Daphnia	*D. magna*	Acute	48	Mortality	NOEC	0.5	nRi3	Re2	(Heinlaan, Ivask et al. 2008)	
Daphnia	*T. platyurus*	Acute	24	Mortality	LC50	2.1	nRi3	Re2	(Heinlaan, Ivask et al. 2008)	
Daphnia	*T. platyurus*	Acute	24	Mortality	NOEC	0.5	nRi3	Re2	(Heinlaan, Ivask et al. 2008)	
Fish	*D. rerio*	Acute	48	Survival	LC50	1.56	nRi2	Re2	(Griffitt, Weil et al. 2007)	
Algae	*N. obtusa*	Chronic	192	Mortality	LC50	2.8	nRi3	Re2	(Manusadzianas, Caillet et al. 2012)	Sonicated suspensions
Algae	*Chlorella*	Acute	0.5	Photosynthesis	IC50	47	nRi3	Re2	(Manusadzianas, Caillet et al. 2012)	Sonicated suspensions
Daphnia	*T. platyurus*	Acute	24	Mortality	LC50	8.5	nRi3	Re2	(Manusadzianas, Caillet et al. 2012)	Sonicated suspensions
Rotifers	*B. calyciflorus*	Acute	24	Mortality	LC50	0.39	nRi3	Re2	(Manusadzianas, Caillet et al. 2012)	Sonicated suspensions
Algae	*N. obtusa*	Chronic	192	Mortality	LC50	4.3	nRi3	Re2	(Manusadzianas, Caillet et al. 2012)	Non-sonicated suspensions
Algae	*Chlorella*	Acute	0.5	Fluorescence	IC50	57	nRi3	Re2	(Manusadzianas, Caillet et al. 2012)	Non-sonicated suspensions
Daphnia	*T. platyurus*	Acute	24	Mortality	LC50	9.3	nRi3	Re2	(Manusadzianas, Caillet et al. 2012)	Non-sonicated suspensions
Rotifers	*B. calyciflorus*	Acute	24	Mortality	LC50	0.24	nRi3	Re2	(Manusadzianas, Caillet et al. 2012)	Non-sonicated suspensions
Daphnia	*C. dubia* neonates	Acute	48	Mortality	LC50	0.419	nRi2	Re2	(Griffitt, Luo et al. 2008)	
Daphnia	*D. pulex* adult	Acute	48	Mortality	LC50	0.06	nRi2	Re2	(Griffitt, Luo et al. 2008)	
Fish	*D. rerio* adult	Acute	48	Mortality	LC50	0.94	nRi2	Re2	(Griffitt, Luo et al. 2008)	
Fish	*D. rerio* juvenile	Acute	48	Mortality	LC50	0.71	nRi2	Re2	(Griffitt, Luo et al. 2008)	
Algae	*P. subcapitata*	Chronic	96	Growth	LC50	0.54	nRi2	Re2	(Griffitt, Luo et al. 2008)	
Algae	*C. reinhardtii*	Chronic	72	Growth	EC50	150.45	nRi2	Re2	(Melegari, Perreault et al. 2013)	
Algae	*C. reinhardtii*	Chronic	72	Growth	NOEC	≤100	nRi2	Re2	(Melegari, Perreault et al. 2013)	
Fish embryos	*D. rerio*	Acute	120	Survival	LC50	>10	nRi2	Re2	(Vicario-Pares, Castanaga et al. 2014)	
Daphnia	*D. magna*	Acute	48	Immobility	EC50	0.32	nRi2	Re1	(Rossetto, Vicentini et al. 2014)	
Daphnia	*D. magna*	Chronic	504	Longevity	NOEC	0.06	nRi2	Re1	(Rossetto, Vicentini et al. 2014)	
Daphnia	*D. magna*	Chronic	504	Growth	NOEC	<0.01	nRi2	Re1	(Rossetto, Vicentini et al. 2014)	
Daphnia	*D. magna*	Chronic	504	Reproduction	NOEC	0.06	nRi2	Re1	(Rossetto, Vicentini et al. 2014)	

Bacteria	*V. fischeri*	Acute	0.25	Luminescence	EC50	7.79	nRi2	Re1	(Rossetto, Vicentini et al. 2014)	
Protozoa	*T. thermophila*	Acute	24	Cell viability	EC50	97.9	nRi2	Re2	(Mortimer, Kasemets et al. 2010)	
Insecta	*A. ligonifer*	Acute	96	Mortality	LC50	569	nRi2	Re2	(Pradhan, Seena et al. 2012)	
Insecta	*A. ligonifer*	Acute	96	Mortality	LOEC	250	nRi2	Re2	(Pradhan, Seena et al. 2012)	
Protozoa	*P. multimicronucleatum*	Acute	48	Mortality	LC50	0.98	nRi2	Re2	(Li, Chen et al. 2012)	
Daphnia	*D. magna*	Acute	72	Mortality	LC50	0.0128	nRi3	Re2	(Fan, Shi et al. 2012)	Octahedral micro/nano Cu_2O
Daphnia	*D. magna*	Acute	72	Mortality	LC50	0.050	nRi3	Re2	(Fan, Shi et al. 2012)	Cubic micro/nano Cu_2O
Daphnia	*D. magna*	Acute	48	Immobilisation	EC50	2.6	nRi3	Re2	(Blinova, Ivask et al. 2010)	
Daphnia	*T. platyurus*	Acute	24	Mortality	LC50	1.7	nRi3	Re2	(Blinova, Ivask et al. 2010)	
Fish	*D. rerio* (larvae)	Acute	96	Mortality	LC50	0.242	nRi3	Re2	(Chen, Zhang et al. 2011)	Cu_2O: LC50 = 242 ppb
Fish	*D. rerio* (larvae)	Acute	96	Gene expression	LOEL	0.121	nRi3	Re2	(Chen, Zhang et al. 2011)	Cu_2O: LOEL = 121 ppb
Fish	*D. rerio* (larvae)	Acute	96	Gene expression	NOEL	0.03	nRi3	Re2	(Chen, Zhang et al. 2011)	Cu_2O: NOEL = 30 ppb
Amphibian	*X. laevis*	Chronic	96	Malformations	TC50	304.25	nRi2	Re2	(Bacchetta, Santo et al. 2012)	<50 nm
Amphibian	*X. laevis*	Chronic	96	Malformations	Significant	10	nRi2	Re2	(Bacchetta, Santo et al. 2012)	<50 nm

Table 20 – Detailed overview of the selected studies on environmental effects of iron (Fe)-containing nanoparticles. Reliability and relevance were assessed according to the article by Hartmann, Lützhøft et al. (In prep) and the associated spreadsheet developed for the scoring of different criteria used to assess the reliability and relevance.

Trophic level	Organism	Acute/chronic	Duration, h	Endpoint	Quantification of endpoint	Value, mg/L	Reliability	Relevance	Reference	Comment
Protozoa	*P. multimicronucleatum*	Acute	48	Mortality	LC50	0.81	nRi2	Re2	(Li, Chen et al. 2012)	Nano-Fe_2O_3
Algae	*P. subcapitata*	Chronic	72	Growth	EC50	7.4	nRi3	Re2	(Markova, Novak et al. 2014)	Iron (II, III) nanoparticles
Bacteria	*S. nidulans*	Chronic	72	Growth	EC50	6.1	nRi3	Re2	(Markova, Novak et al. 2014)	Iron (II, III) nanoparticles
Daphnia	*D. magna*	Acute	48	Immobilization	EC50	16.6	nRi3	Re2	(Markova, Novak et al. 2014)	Iron (II, III) nanoparticles
Daphnia	*D. magna*	Acute	48	Immobilization	EC50	>1000	nRi2	Re1	(Marsalek, Jancula et al. 2012)	Suspension (Nanofer 25)
Fish	*P. reticulate*	Acute	96	Mortality	EC50	>2500	nRi2	Re1	(Marsalek, Jancula et al. 2012)	Suspension (Nanofer 25)
Bacteria	*M. aeruginosa*	Acute	24	Biomass removal	EC50	50	nRi2	Re1	(Marsalek, Jancula et al. 2012)	Suspension (Nanofer 25)
Algae	*I. galbana**	Chronic	96	Growth	Significant	3.1	nRi2	Re1	(Keller, Garner et al. 2012)	Suspension (PGSM coated Nanofer 25S)
Algae	*D. tertiolecta**	Chronic	96	Growth	Significant	1.3	nRi2	Re1	(Keller, Garner et al. 2012)	Suspension (PGSM coated Nanofer 25S)
Algae	*T. pseudonana**	Chronic	96	Growth	Significant	0.42	nRi2	Re1	(Keller, Garner et al. 2012)	Suspension (PGSM coated Nanofer 25S)
Algae	*P. subcapitata*	Chronic	96	Growth	Significant	8.24	nRi2	Re1	(Keller, Garner et al. 2012)	Suspension (PGSM coated Nanofer 25S)
Daphnia	*D. magna*	Acute	96	Survival	Significant	0.5	nRi2	Re1	(Keller, Garner et al. 2012)	Suspension (PGSM coated Nanofer 25S)
Daphnia	*D. magna*	Acute	48	Mortality	EC50	0.23	nRi3	Re1	(Garcia, Espinosa et al. 2011)	Nano-Fe_3O_4
Bacteria	*V. fischeri*	Acute	0.25	Mortality	EC50	240	nRi3	Re1	(Garcia, Espinosa et al. 2011)	Nano-Fe_3O_4
Bacteria	*V. fischeri*	Acute	0.25	Luminescence	IC50	35.2	nRi3	Re2	(Recillas, Garcia et al. 2011)	Nano-Fe_3O_4
Daphnia	*D. magna*	Acute	96	Immobilization	EC50	27.9	nRi2	Re2	(Baumann, Koeser et al. 2014)	Nano-Fe_3O_4 coated with Dextran
Fish, Embryo	*D. rerio*	Acute	168	Survival	NOEC	<50	nRi2	Re2	(Zhu, Tian et al. 2012)	Nano-Fe_2O_3
Fish, Embryo	*D. rerio*	Acute	168	Survival	LC50	53.35	nRi2	Re2	(Zhu, Tian et al. 2012)	Nano-Fe_2O_3
Fish, Embryo	*D. rerio*	Acute	168	Hatching rate	NOEC	10	nRi2	Re2	(Zhu, Tian et al. 2012)	Nano-Fe_2O_3
Fish, Embryo	*D. rerio*	Acute	168	Hatching rate	EC50	36.06	nRi2	Re2	(Zhu, Tian et al. 2012)	Nano-Fe_2O_3

* Marine organism, tests made in seawater; PGSM: Polyethylene Glycol Sorbitan Monostearate

Table 21 – Detailed overview of the selected studies on environmental effects of cerium oxide (CeO_2) nanoparticles. Reliability and relevance were assessed according to the article by Hartmann, Lützhøft et al. (In prep) and the associated spreadsheet developed for the scoring of different criteria used to assess the reliability and relevance.

Trophic level	Organism	Acute/chronic	Duration, h	Endpoint	Quantification of endpoint	Value, mg/L	Reliability	Relevance	Reference	Comment
Protozoa	*P. multimicronucleatum*	Acute	48	Mortality	LC50	1832.5	nRi2	Re2	(Li, Chen et al. 2012)	
Bacteria	*V. fischeri*	Acute	0.25	Luminescence	EC50	>100	nRi3	Re2	(Velzeboer, Hendriks et al. 2008)	
Algae	*P. subcapitata*	Acute	4.5	Photosynthesis	EC50	>100	nRi3	Re2	(Velzeboer, Hendriks et al. 2008)	
Crustacean	*S. sphaericus*	Acute	48	Mortality	EC50	>100	nRi3	Re2	(Velzeboer, Hendriks et al. 2008)	
Daphnia	*D. magna*	Acute	96	Mortality	LC50	>10	nRi2	Re1	(Gaiser, Fernandes et al. 2012)	
Daphnia	*D. magna*	Chronic	504	Mortality	LC50	<10	nRi2	Re1	(Gaiser, Fernandes et al. 2012)	
Algae	*P. subcapitata*	Chronic	72	Growth inhibition	NOEC	3.2	nRi2	Re2	(Van Hoecke, Quik et al. 2009)	
Algae	*P. subcapitata*	Chronic	72	Growth inhibition	LOEC	5.6	nRi2	Re2	(Van Hoecke, Quik et al. 2009)	
Algae	*P. subcapitata*	Chronic	72	Growth inhibition	EC10	3.4	nRi2	Re2	(Van Hoecke, Quik et al. 2009)	
Algae	*P. subcapitata*	Chronic	72	Growth inhibition	EC50	11.7	nRi2	Re2	(Van Hoecke, Quik et al. 2009)	
Daphnia	*T. platyurus*	Acute	24	Mortality	NOEC	>5000	nRi2	Re2	(Van Hoecke, Quik et al. 2009)	
Daphnia	*D. magna*	Actue	48	Immobility	NOEC	>1000	nRi2	Re2	(Van Hoecke, Quik et al. 2009)	
Daphnia	*D. magna*	Chronic	504	Survival	NOEC	32	nRi2	Re2	(Van Hoecke, Quik et al. 2009)	
Daphnia	*D. magna*	Chronic	504	Survival	LOEC	56	nRi2	Re2	(Van Hoecke, Quik et al. 2009)	
Daphnia	*D. magna*	Chronic	504	Survival	EC50	40.7	nRi2	Re2	(Van Hoecke, Quik et al. 2009)	
Daphnia	*D. magna*	Chronic	504	Reproduction	NOEC	<18	nRi2	Re2	(Van Hoecke, Quik et al. 2009)	
Daphnia	*D. magna*	Chronic	504	Reproduction	LOEC	18	nRi2	Re2	(Van Hoecke, Quik et al. 2009)	
Daphnia	*D. magna*	Chronic	504	Reproduction	EC10	8.8	nRi2	Re2	(Van Hoecke, Quik et al. 2009)	
Daphnia	*D. magna*	Chronic	504	Reproduction	EC50	20.5	nRi2	Re2	(Van Hoecke, Quik et al. 2009)	
Fish embryo	*D. rerio*	Acute	72	Mortality	NOEC	>200	nRi2	Re2	(Van Hoecke, Quik et al. 2009)	
Fish embryo	*D. rerio*	Acute	72	Hatching	NOEC	>200	nRi2	Re2	(Van Hoecke, Quik et al. 2009)	
Fish embryo	*D. rerio*	Acute	72	Malformation	NOEC	>200	nRi2	Re2	(Van Hoecke, Quik et al. 2009)	
Bacteria	*Anabaena*	Acute	24	Luminescence	EC50	0.27	nRi3	Re2	(Rodea-Palomares, Boltes et al. 2011)	
Algae	*P. subcapitata*	Chronic	72	ATP	EC50	2.4	nRi3	Re2	(Rodea-Palomares, Boltes et al. 2011)	
Algae	*P. subcapitata*	Chronic	72	Growth	EC50	0.88	nRi3	Re2	(Rodea-Palomares, Boltes et al. 2011)	
Algae	*P. subcapitata*	Chronic	96	Growth	IC50	10.3	nRi2	Re2	(Rogers, Franklin et al. 2010)	
Algae	*P. subcapitata*	Chronic	72	Growth	EC10	0.7	nRi2	Re2	(Manier, Bado-Nilles et al. 2013)	
Algae	*P. subcapitata*	Chronic	72	Growth	EC50	5.6	nRi2	Re2	(Manier, Bado-Nilles et al. 2013)	
Bacteria	*V. fischeri*	Acute	0.25	Luminescence	IC50	35.2	nRi3	Re2	(Recillas, Garcia et al. 2011)	
Daphnia	*D. similis*	Acute	48	Immobility	EC50	0.26	nRi2	Re2	(Artells, Issartel et al. 2013)	
Daphnia	*D. pulex*	Acute	48	Immobility	EC50	91.79	nRi2	Re2	(Artells, Issartel et al. 2013)	
Daphnia	*D. pulex*	Acute	72	Immobility	EC50	0.94	nRi2	Re2	(Artells, Issartel et al. 2013)	

Daphnia	*D. pulex*	Acute	96	Immobility	EC50	0.78	nRi2	Re2	(Artells, Issartel et al. 2013)
Fish embryo	*D. rerio*	Acute	72	Mortality	LC50	>2000	nRi2	Re1	(Felix, Ortega et al. 2013)

Table 22 – Detailed overview of the selected studies on environmental effects of quantum dots (QDs) nanoparticles. Reliability and relevance were assessed according to the article by Hartmann, Lützhøft et al. (In prep) and the associated spreadsheet developed for the scoring of different criteria used to assess the reliability and relevance.

Trophic level	Organism	Acute/chronic	Duration, h	Endpoint	Quantification of endpoint	Value, mg/L	Reliability	Relevance	Reference	Comment
Algae	*P. subcapitata*	Chronic	96	Growth inhibition	EC50	0,0371	nRi3	Re2	(Bouldin, Ingle et al. 2008)	Based on the NP
Algae	*P. subcapitata*	Chronic	96	Growth inhibition	EC50	9,638	nRi3	Re2	(Bouldin, Ingle et al. 2008)	Based on Cd
Algae	*P. subcapitata*	Chronic	96	Growth inhibition	EC50	2,410	nRi3	Re2	(Bouldin, Ingle et al. 2008)	Based on Se
Daphnia	*C. dubia*	Acute	48	Mortality	LC50	0,110	nRi3	Re2	(Bouldin, Ingle et al. 2008)	Based on the NP
Daphnia	*C. dubia*	Acute	48	Mortality	LC50	28,6	nRi3	Re2	(Bouldin, Ingle et al. 2008)	Based on Cd
Daphnia	*C. dubia*	Acute	48	Mortality	LC50	7,15	nRi3	Re2	(Bouldin, Ingle et al. 2008)	Based on Se
Algae	*C. reinhardtii*	Chronic	72	Growth inhibition	EC50	5	nRi2	Re2	(Wang, Zhang et al. 2008)	Freshwater, based on the NP
Algae	*C. reinhardtii*	Acute	3	Stress response genes	Significant	0,1	nRi2	Re2	(Wang, Zhang et al. 2008)	Freshwater, based on the NP
Daphnia	*D. magna*	Acute	48	Mortality	EC50	>2,5*	nRi2	Re2	(Lee, Kim et al. 2009)	White FLU, MPA coating
Daphnia	*D. magna*	Acute	96	Mortality	EC50	NC	nRi2	Re2	(Lee, Kim et al. 2009)	White FLU, MPA coating
Daphnia	*D. magna*	Acute	48	Mortality	EC50	0,392*	nRi2	Re2	(Lee, Kim et al. 2009)	UV-B, MPA coating
Daphnia	*D. magna*	Acute	96	Mortality	EC50	0,056*	nRi2	Re2	(Lee, Kim et al. 2009)	UV-B, MPA coating
Daphnia	*D. magna*	Acute	48	Mortality	EC50	0,385*	nRi2	Re2	(Lee, Kim et al. 2009)	Sunlight, MPA coating
Daphnia	*D. magna*	Acute	96	Mortality	EC50	0,028*	nRi2	Re2	(Lee, Kim et al. 2009)	Sunlight, MPA coating
Daphnia	*D. magna*	Acute	48	Mortality	EC50	0,067*	nRi2	Re2	(Lee, Kim et al. 2009)	White FLU, GA coating
Daphnia	*D. magna*	Acute	96	Mortality	EC50	0,028*	nRi2	Re2	(Lee, Kim et al. 2009)	White FLU, GA coating
Daphnia	*D. magna*	Acute	48	Mortality	EC50	0,051*	nRi2	Re2	(Lee, Kim et al. 2009)	UV-B, GA coating
Daphnia	*D. magna*	Acute	96	Mortality	EC50	0,008*	nRi2	Re2	(Lee, Kim et al. 2009)	UV-B, GA coating
Daphnia	*D. magna*	Acute	48	Mortality	EC50	0,011*	nRi2	Re2	(Lee, Kim et al. 2009)	Sunlight, GA coating
Daphnia	*D. magna*	Acute	96	Mortality	EC50	0,001*	nRi2	Re2	(Lee, Kim et al. 2009)	Sunlight, GA coating
Daphnia	*D. magna*	Acute	48	Mortality	EC50	-	nRi2	Re2	(Kim, Park et al. 2010)	White FLU, MPA coating
Daphnia	*D. magna*	Acute	48	Mortality	EC50	-	nRi2	Re2	(Kim, Park et al. 2010)	UV-B, MPA coating
Daphnia	*D. magna*	Acute	48	Mortality	EC50	0,096*	nRi2	Re2	(Kim, Park et al. 2010)	White FLU, GA coating
Daphnia	*D. magna*	Acute	48	Mortality	EC50	0,059*	nRi2	Re2	(Kim, Park et al. 2010)	UV-B, GA coating
Daphnia	*D. magna*	Acute	48	ROS	Significant	0,030*	nRi2	Re2	(Kim, Park et al. 2010)	UV-B, GA coating
Daphnia	*D. magna*	Acute	48	mRNA hemoglobin	Significant	0,030*	nRi2	Re2	(Kim, Park et al. 2010)	GA coating
Daphnia	*D. magna*	Acute	48	mRNA	Significant	0,003*	nRi2	Re2	(Kim, Park et al. 2010)	UV-B, MPA coating
Nematodes	*C. elegans*	Acute	72	Lifespan	Significant	300	nRi3	Re2	(Contreras, Cho et al. 2013)	Terrestrial, core QD
Nematodes	*C. elegans*	Chronic	312#	Brood size	Significant	20	nRi3	Re2	(Contreras, Cho et al. 2013)	Terrestrial, core QD
Nematodes	*C. elegans*	Chronic	288#	Only 1. G brood size	Significant	50	nRi3	Re2	(Contreras, Cho et al. 2013)	Terrestrial, core QD
Nematodes	*C. elegans*	Chronic	288#	All 4 Gs brood size	Significant	100	nRi3	Re2	(Contreras, Cho et al. 2013)	Terrestrial, core QD
Nematodes	*C. elegans*	Chronic	336#	Only 2. G length	Significant	10	nRi3	Re2	(Contreras, Cho et al. 2013)	Terrestrial, core QD

Nematodes	*C. elegans*	Chronic	288#	Only 1. G length	Significant	100	nRi3	Re2	(Contreras, Cho et al. 2013)	Terrestrial, core QD
Nematodes	*C. elegans*	Chronic	336#	From 2. G locomotor	Significant	10	nRi3	Re2	(Contreras, Cho et al. 2013)	Terrestrial, core QD
Nematodes	*C. elegans*	Chronic	288#	Only 1. G length	Significant	50	nRi3	Re2	(Contreras, Cho et al. 2013)	Terrestrial, core-shell QD
Nematodes	*C. elegans*	Chronic	288#	Only 1. G length	Significant	100	nRi3	Re2	(Contreras, Cho et al. 2013)	Terrestrial, core-shell QD
Bacteria	*E. coli*	Acute	Ns	β-galactosidase	34% inhibition	0,01-100	nRi2	Re2	(Pokhrel, Silva et al. 2012)	Soil, octacecylamine coating

*: Corresponding to the free Cd^{2+} ion concentration in order to compare with $CdCl_2$/$CdSO_4$ toxicity studies; GA: Gum arabicum; MPA: Mercaptoproprionic acid; #: Not directly specified in the article, but assumed from the text and figures; NC: not calculated; -: No mortality observed; G: generation; core QD: CdSe; core-shell QD: CdSe/ZnS; Ns: Not stated;

Table 23 – Detailed overview of the selected studies on environmental effects of carbon black (CB) nanoparticles. Reliability and relevance were assessed according to the article by Hartmann, Lützhøft et al. (In prep) and the associated spreadsheet developed for the scoring of different criteria used to assess the reliability and relevance.

Trophic level	Organism	Acute/chronic	Duration, h	Endpoint	Quantification of endpoint	Value, mg/L	Reliability	Relevance	Reference	Comment
Daphnia	*A. amphitrite* naupli	Acute	48	Mortality	LC50	1840	nRi3	Re2	(Mesaric, Sepcic et al. 2013)	Marine, 13 nm, as received
Daphnia	*A. amphitrite* naupli	Acute	48	Swim speed inhibition	EC50	480	nRi3	Re2	(Mesaric, Sepcic et al. 2013)	Marine, 13 nm, as received
Daphnia	*A. amphitrite* cyprids	Acute	72	Settlement inhibition	EC50	30	nRi3	Re2	(Mesaric, Sepcic et al. 2013)	Marine, 13 nm, as received
Daphnia	*A. franciscana larvae*	Acute	24	Mortality	LC50	370	nRi3	Re1	(Rodd, Creighton et al. 2014)	Marine, as produced
Daphnia	*A. franciscana larvae*	Acute	24	Mortality	LC50	1000*	nRi3	Re1	(Rodd, Creighton et al. 2014)	Marine, hydrophobic
Daphnia	*A. franciscana larvae*	Acute	24	Mortality	LC50	>1000*	nRi3	Re1	(Rodd, Creighton et al. 2014)	Marine, hydrophilic
Daphnia	*A. franciscana larvae*	Acute	24	HSP70 increase	Significant	50	nRi3	Re1	(Rodd, Creighton et al. 2014)	Marine, as produced
Daphnia	*A. franciscana larvae*	Acute	24	HSP70 increase	Significant	50	nRi3	Re1	(Rodd, Creighton et al. 2014)	Marine, hydrophobic
Daphnia	*A. franciscana larvae*	Acute	24	HSP70 increase	Not significant	50	nRi3	Re1	(Rodd, Creighton et al. 2014)	Marine, hydrophilic
Molluscs	*M. galloprovincialis* LAM hemocyte	Acute	0,5	Membrane stability	Not significant	10	nRi3	Re2	(Canesi, Ciacci et al. 2008)	Marine, 35 and 400 nm
Molluscs	*M. galloprovincialis* LAM hemocyte	Acute	0,5	Lysozyme release	Significant	1	nRi3	Re2	(Canesi, Ciacci et al. 2008)	Marine, 35 and 400 nm
Molluscs	*M. galloprovincialis* LAM hemocyte	Acute	0,5	ROS	Significant	1	nRi3	Re2	(Canesi, Ciacci et al. 2008)	Marine, 35 and 400 nm
Molluscs	*M. galloprovincialis* LAM hemocyte	Acute	1	Nitrite production	Significant	1	nRi3	Re2	(Canesi, Ciacci et al. 2008)	Marine, 35 and 400 nm
Molluscs	*M. galloprovincialis* LAM hemocyte	Acute	0,75	Mitochondria	Significant	10	nRi3	Re2	(Canesi, Ciacci et al. 2008)	Marine, 35 and 400 nm
Molluscs	*M. galloprovincialis* LAM hemocyte	Acute	0,08	MAPK	Significant	10	nRi3	Re2	(Canesi, Ciacci et al. 2008)	Marine, 35 and 400 nm
Flies	*D. melanogaster* larvae	Chronic	48	Hatching	Not significant	1000#	nRi3	Re2	(Liu, Vinson et al. 2009)	Terrestrial
Flies	*D. melanogaster* adult	Chronic	48	Survival	Significant	3,3¤	nRi3	Re2	(Liu, Vinson et al. 2009)	Terrestrial
Flies	*D. melanogaster* adult	Ns	Ns	Locomotor activity	Significant	3,3¤	nRi3	Re2	(Liu, Vinson et al. 2009)	Terrestrial

ROS: Reactive oxygen species; MAPK: Mitogen activated protein kinase; *: It seems like in the text that the authors have mixed up these two nanoparticle modifications, however, as the effect concentration is this high and at the same level, it is not judged crucial, compared with the overall assessment of an nRi3 reliability study; #: µg NM per g food; ¤: Total dose in vial in mg.

Appendix 2: ECHA Part R.10: Table R.10-4 Assessment factor to derive a $PNEC_{aquatic}$.

Table R.10-4 Assessment factors to derive a $PNEC_{aquatic}$

Available data	Assessment factor
At least one short-term L(E)C50 from each of three trophic levels (fish, invertebrates (preferred Daphnia) and algae)	1000 [a)]
One long-term EC10 or NOEC (either fish or Daphnia)	100 [b)]
Two long-term results (e.g. EC10 or NOECs) from species representing two trophic levels (fish and/or Daphnia and/or algae)	50 [c)]
Long-term results (e.g. EC10 or NOECs) from at least three species (normally fish, Daphnia and algae) representing three trophic levels	10 [d)]
Species sensitivity distribution (SSD) method	5-1 (to be fully justified case by case) [e)]
Field data or model ecosystems	Reviewed on a case by case basis [f)]

Notes to Table R.10-4:

a) The use of a factor of 1000 on short-term toxicity data is a conservative and protective factor and is designed to ensure that substances with the potential to cause adverse effects are identified in the hazard assessment. It assumes that each of the uncertainties identified above makes a significant contribution to the overall uncertainty. For any given substance there may be evidence that this is not so, or that one particular component of the uncertainty is more important than any other. In these circumstances it may be necessary to vary this factor. This variation may lead to a raised or lowered assessment factor depending on the available evidence. A factor lower than 100 should not be used in deriving a $PNEC_{water}$ from short-term toxicity data except for substances with intermittent release (see Section R.10.3.3.).

Variation from a factor of 1000 should not be regarded as normal and should be fully supported by accompanying evidence.

b) An assessment factor of 100 applies to a single long-term result (e.g. EC10 or NOECs) (fish or Daphnia) if this result was generated for the trophic level showing the lowest L(E)C50 in the short-term tests.

If the only available long-term result (e.g. EC10 or NOECs)is from a species (standard or non-standard organism) which does not have the lowest L(E)C50 from the short-term tests, it cannot be regarded as protective of other more sensitive species using the assessment factors available. Thus the hazard assessment is based on the short-term data with an assessment factor of 1000. However, the resulting PNEC based on short-term data may not be higher than the PNEC based on the long-term result available.

An assessment factor of 100 applies also to the lowest of two long-term results (e.g. EC10 or NOECs) covering two trophic levels when such results have not been generated from that showing the lowest L(E)C50 of the short-term tests. This should, however, not apply in cases where the acutely most sensitive species has an L(E)C50 value lower than the lowest long term result (e.g. EC10 or NOECs) value. In such cases the PNEC might be derived by using an assessment factor of 100 to the lowest L(E)C50 of the short-term tests.

c) An assessment factor of 50 applies to the lowest of two long term results (e.g. EC10 or NOECs) covering two trophic levels when such results have been generated covering that level showing the lowest L(E)C50 in the short-term tests. It also applies to the lowest of three long term results (e.g. EC10 or NOECs) covering three trophic levels when such results have not been generated from that trophic level showing the lowest L(E)C50 in the short-term tests. This should however not apply in cases where the acutely most sensitive species has an L(E)C50 value lower than the lowest long term result (e.g. EC10 or NOECs) value. In such cases the PNEC might be derived by using an assessment factor of 100 to the lowest L(E)C50 of the short-term tests.

d) An assessment factor of 10 will normally only be applied when long-term toxicity results (e.g. EC10 or NOECs) are available from at least three species across three trophic levels (e.g. fish, Daphnia, and algae or a non-standard organism instead of a standard organism).

When examining the results of long-term toxicity studies, the PNECwater should be calculated from the lowest available long term result. Extrapolation to the ecosystem effects can be made with much greater confidence, and thus a reduction of the assessment factor to 10 is possible. This is only sufficient, however, if the species tested can be considered to represent one of the more sensitive groups. This would normally only be possible to determine if data were available on at least three species across three trophic levels.

Appendix 3: Guidance text according to (Moermond, Kase et al. In prep) on criteria used to evaluate reliability of an effect study on ENMs (referring to criteria numbers in Table 9).

Physico-chemical parameters – As for chemicals in general, knowledge on physico-chemical parameters of the nanomaterial are required to enable a correct evaluation of the test reliability. In contrast to conventional chemicals, however, it is generally not possible to obtain this information from chemical handbooks as a CAS number and/or the chemical composition is not sufficient to describe and identify the nanomaterial. For example many physico-chemical parameters will depend on factors such as particle size or coating material. This information should be given in the study documentation.

Before evaluating the test, check the physico-chemical characteristics of the nanomaterial. Check the core chemical composition, primary particle size, surface chemistry (coating material, functionalization etc. if used). Other important information include solubility of the nanomaterial (check if the nanomaterial is likely to undergo dissolution and if the dissolved species have a known ecotoxicological effect). If the nanomaterial is coated and/or stabilised, what are the known properties of the coating material? Is it photocatalytic or reactive? What is the agglomeration/aggregation state of the particles? What is the specific surface area? **If the reported information on physicochemical properties of the nanomaterial is considered to be insufficient to evaluate the reliability, the study should be given the score nRi4.**

1 - Consider if a sufficiently detailed description of endpoints and methodology is available? (▲▲)
It should be evaluated if the study methodology and endpoints are generally described in a clear and transparent manner. For ecotoxicity data in general (see also (Moermond, Kase et al. In prep)), and nano-ecotoxicity data in particular, it is not sufficient to state that a certain guideline has been followed. The specific details of the test methodology should be provided in the study. At present there are no specific OECD test guidelines available for ecotoxicity testing of nanomaterials and the current guideline methodologies are therefore often (at least to some extend) adapted to nanomaterial testing especially with respect to preparation of test suspensions. Due to the great variety of nanomaterial properties it is highly unlikely that a completely harmonised guideline for nanomaterial dispersion will ever become available covering all types of nanomaterials. This implies that some degree of case-by-case methodologies will be the reality, emphasising the need for clear and transparent reporting. For example, when testing nanomaterials even little changes to the sample preparation and test methodology can have a great impact on the nanomaterial behaviour in the test system and resulting effects. It is important to know exactly how the stock dispersion was made (including e.g. nanomaterial concentration, sonication time, sonication energy, volume and material of suspension vessel, pre-wetting steps, addition of dispersants etc.) as this will directly influence the characteristics of the stock suspension and hence the subsequent exposure conditions. **If the description of the study is not sufficiently detailed to evaluate reliability, the study should be assigned nRi4.**

2 - Is a standard method (e.g., OECD, ISO) or modified standard used? (Δ)
As for conventional chemicals the use of standard methods is not in itself a guarantee for study reliability. It may ensure better and more complete documentation but does not *per se* ensure e.g. an appropriate study design or correct interpretation of test results (Moermond, Kase et al. In prep). Unless specific standard methods have been developed explicitly for nanomaterials, the use of standard methods may on the contrary result in lower reliability. This is due to the fact that current guidelines are developed mainly for soluble chemicals and do not take into account the particulate nature of nanomaterials. Non-standard tests – or modified guideline tests – may therefore be equally or more reliable compared to standard guideline studies. A well-considered modification of a standard method is preferred over uncritically applying a standard method that does not account for the nano-specific physical chemical properties. **The use of standard guidelines is therefore not considered to be a critical criterion for reliability *per se*.**

3 - Is the test performed under GLP conditions? (-)
As for standard guidelines, GLP helps to ensure reproducibility and transparency but is not in itself a guarantee for reliability. **The use of GLP is therefore not considered to be a critical criterion for reliability.**

4 - If applicable, are validity criteria fulfilled (e.g. control survival, growth)? (Δ/▲)
This criterion applies equally to conventional chemicals and nanomaterials. Such criteria especially apply to studies that follow guidelines or modified guidelines containing requirements regarding validity criteria. However, any study where a smaller or larger part of the control organisms die during test performance may constitute a test set-up problem, leading to a test artefact. If the guidelines have been modified then the original validity criteria may not be relevant or possible to achieve. In these cases expert judgement is required. **For a detailed description see** (Moermond, Kase et al. In prep).

5 - Are appropriate controls performed (e.g. dispersant control, metal ion control, negative and positive control)? (▲)
For conventional chemicals it is common to use solvents to dissolve poorly soluble compounds. For nanomaterials it is not feasible and/or relevant to dissolve the particles. Instead the aim is rather to achieve a stable and homogeneous suspension. For example, when testing silver nanoparticles, the aim is not to dissolve the particles into ions and ion complexes but rather to test silver in its nano-particulate form. Stable and homogeneous dispersions can sometimes be achieved by adding a dispersant or a stabiliser to the media. This could be a substance that adsorbs to the surface of the particle and causes electrostatic or steric stabilisation. It is important to include dispersant controls to ensure that this substance does not in itself cause toxic effects. **If a dispersant has been used, but no information is provided regarding its ecotoxicity, the study is considered not to be reliable (nRi3).**

Some nanomaterials will dissolve over time. This is for example the case for silver and zinc nanoparticles. Other materials are more inert (e.g. TiO_2). For nanomaterials that release metal ions it is important that the study also addresses the metal ion contribution to the observed toxicity. This is often done by including metal ion controls and monitoring the ion release from the nanomaterial. The key in data interpretation is then to evaluate the particle as well as the ion contributions to the observed ecotoxicological effects. There are also other ways of addressing this issue such as biomarkers etc. Expert judgement is therefore needed to evaluate if the study is lacking critical information regarding metal ion controls.

A negative control refers to test samples in pure media without the presence of nanomaterials, dispersants etc. **Mortality in the negative controls may indicate some problems with the study reliability and result in a lower reliability score (nRi2 or nRi3) subject to expert judgement**. Positive controls are not considered critical to the reliability of a study.

6 - Is the test substance identified with name or CAS-number? Are nanomaterial characteristics reported that allow for a clear identification of the tested material (e.g. particle size, shape, particle size distribution, surface area)? Are test results reported for the appropriate compound? (▲▲)
Contrary to conventional chemicals it is not sufficient to specify name or CAS-number for nanomaterials as they may cover a wide range of nanomaterials with chemical composition as the only common denominator. Nanomaterials should not only be identified from their chemical composition but also from other physico-chemical properties such as primary particle sizes, shape, crystal structure (relevant for e.g. TiO_2), presence of coatings etc. Hence, information should be available that clearly and unambiguously identifies the nanomaterial for which the ecotoxicity data has been established. The detail of this information should make it possible to compare the specific

tested substance with the substance for which e.g. the risk assessment is to be performed. The following parameters are considered to be the minimum characterization requirements that will allow for unambiguous substance identification:

- core chemical composition,
- purity,
- primary particle size (measured),
- shape,
- crystal structure (if relevant),
- radiolabelled (if used),
- surface chemistry (coating material, functionalization etc. if used).

If the information is insufficient (generic and e.g. reporting only the chemical composition) the study is considered not to be reliable (nRi3).

7 - Is the purity of the test substance reported? This includes information on synthesis by-products as well as synthesis catalysts and presence of other crystalline forms of the substance. And/or, is the source of the test substance trustworthy? (▲)

As for conventional chemicals it is important that tested nanomaterials are of high purity and that toxic effects attributed to impurities are accounted for. For nanomaterials impurities may be formed as a result of synthesis. For example carbon nanotubes (CNTs) may contain metal impurities that will contribute to the overall toxicity. Also, different crystalline forms of the substance may be formed during the synthesis resulting in an untended fraction of another crystalline form. This is for example the case for TiO_2 where the anatase form may contain fractions of rutile and amorphous particles. **If, based on expert judgement, it is evaluated that the tested nanomaterial is likely to contain impurities, but this is not reported in the study report, the reliability of the study should be lowered.**

8 - If a formulation is used or if impurities or coatings are present: Do other ingredients in the formulation, the impurities or the coatings exert an effect? Is the amount of test substance in the formulation known? (Δ/▲)

When impurities are reported or known to be present, or if the nanomaterial is coated (for example to stabilise it in a dispersion), the potential negative (or positive) effects of the impurities and/or coating should be addressed. This should clarify if the observed effects can be partly or fully attributed to the impurities and/or the coating material. **If such information is not provided then the reliability of the study should be lowered. For uncoated nanomaterials with a high purity this is however not a critical criteria.**

9 - Are the organisms well described (e.g. scientific name, sex, weight, length, growth, age/life stage, strain/clone, gender if appropriate)? (▲)

This criterion does not differ from conventional chemicals. **For a detailed description see** (Moermond, Kase et al. In prep).

10 - Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms been pre-exposed to test compound or other unintended stressors? (▲▲)

This criterion does not differ from conventional chemicals. **For a detailed description see** (Moermond, Kase et al. In prep)**.** Pre-exposure of the organism to other stressors obviously lowers the study reliability. It should be ensured that the organisms are fit prior to nanomaterial exposure.

11 - Is the experimental test system, test design and test vessel scientifically appropriate for testing of nanomaterials (e.g., static, flow-through, renewal; light/dark conditions; open/closed systems; still/stirred; exposure route)? (▲▲)

As for conventional chemicals the experimental system should be appropriate for the test substance in terms of e.g. vessel materials and use of methods that prevent loss of the test substance from the

system (see (Moermond, Kase et al. In prep)). For nanomaterials it is often observed that the concentration in the water phase will decrease significantly during the test due to particle aggregation/agglomeration and subsequent settling in the test vials. Discussions are ongoing, e.g. within the OECD WPMN and an OECD Expert Group whether this has to be accepted for nanomaterials, whether a higher loss should be acceptable, whether the dispersions have to be stabilised or whether non-guideline aquatic test systems are more appropriate. However, so far there is no clear consensus on this matter and **the appropriateness of the applied methods with regards to testing of nanomaterials will have to be evaluated based on expert judgement. If the method is considered not being appropriate the study should be given reliability nRi3.**

12- Is the experimental system appropriate for the test organism; e.g., choice of medium or test water, feeding, water characteristics, temperature, light/dark conditions, pH, oxygen content? Have conditions been stable during the test? (▲▲)
This criterion does not differ from conventional chemicals. **For a detailed description see** (Moermond, Kase et al. In prep).

13 – Were exposure concentrations stable throughout the duration of the test (taking the use of a dispersant/stabilizer/solvent into account)? And was the exposure stable in qualitatively terms? If not, has this been accounted for in the data interpretation? If a dispersant/stabilizer/solvent is used, is the dispersant/stabilizer/solvent within the appropriate range and is a dispersant/stabilizer/solvent control included? (▲)
Achieving a stable nanomaterial test suspension is a challenging task as nanomaterials tend to agglomerate and sediment in test media. This process is affected by factors such as media composition, ionic strength, and pH. The introduction of a test organism is known to further destabilise the test suspension, leading to unstable exposure conditions during the tests. In addition to quantitative (concentration) changes, exposure may also change qualitatively due to nanomaterial transformation processes. This makes it very important to monitor nanomaterial properties (e.g. agglomeration, dissolution, and degradation), settling behaviour and water phase concentrations during aquatic tests. For conventional chemicals the OECD operates with validity criteria of max 20% loss of the test substance during a test. If the test concentration varies more than ±20% over the duration of the test this excludes the use of initial concentrations as a measure of exposure. Instead concentrations should be based on measurements throughout the test. Discussions are ongoing if such criteria are relevant for tests with nanomaterials (Kennedy et al., 2015). Nonetheless it is important that exposure concentrations are monitored throughout the duration of the test and that its impact on observed effects is discussed. If a dispersant/stabiliser/solvent has been used to achieve a stable dispersion the potential positive or adverse effects of this substance on the overall ecotoxicity should be addressed. **If such information is not provided then the reliability of the study should be lowered**

14 - Is a correct spacing between exposure concentrations applied? (Δ)
Exposure concentrations can be controversial when it comes to nanomaterials. Firstly there may be a correlation between nanomaterial concentration and agglomeration behaviour, meaning that exposure changes qualitatively along the dose-axis in a dose-response curve. Secondly, it has been suggested that concentration may not (alone) be sufficient as a dose-descriptor and other dose-metrics (such as surface area) have been proposed. It may therefore be very challenging to even define a 'correct spacing' between exposure concentrations. **This criterion is therefore considered not to be critical for the reliability of ecotoxicity studies of nanomaterials.** Expert judgement should however be applied to ensure that the exposure concentrations are chosen in a reasonable manner.

15 - Is the exposure duration defined? (▲▲)

This criteria does not differ from conventional chemicals. **For a detailed description see** (Moermond, Kase et al. In prep).

16 - Have analyses been performed to verify exposure, e.g. substance concentrations and physico-chemical transformations of the test substance over the duration of the test? (▲▲)
This criterion is linked to criterion 13 above. As mentioned it is important to monitor nanomaterial exposure over the duration of the test: not only quantitatively but also qualitatively. Important papameters to monitor *over the duration of the* test include:

- *agglomeration state*
- *particle size distribution*
- *surface charge/zeta potential*
- *ion release*

Preferably this information should be available for all tested concentrations and measured at regular intervals.

17 - Is the biomass loading of the organisms in the test system within the appropriate range (< 1 g/L)? (▲)
For nanomaterials physical 'overload' phenomena are sometimes observed and it is thus important to evaluate if the effects on the biomass is caused by test artefacts. This requires an expert evaluation of the nanomaterial loading possibly combined with images of the biomass in the test system. For algal growth inhibition tests shading effects may be relevant. If there is a clear indication of test artefacts caused by a high nanomaterial loading then this with negatively impact reliability.

18 – Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations? (▲)
This criteria does not differ from conventional chemicals. **For a detailed description see** (Moermond, Kase et al. In prep).

19 - Are appropriate statistical methods used? (▲)
This criterion does not differ from conventional chemicals. **For a detailed description see** (Moermond, Kase et al. In prep).

20 - Is a dose response curve observed? Is the response statistically significant? (-)
See explanation to criterion 14. There may be inherent characteristics on the nanomaterial test system which impedes the establishment of dose response curves. **This criterion is therefore considered not to be critical for the reliability of ecotoxicity studies of nanomaterials.** Expert judgement should however be applied to evaluate the test results and the statistical significance of the observed response.

21 - Is sufficient data available to check the calculation of endpoints and validity criteria (e.g., control data, dose-response curves)?
This may refer for example to the access to raw data. It is important to be able to verify the data presented. However, full access to all raw data is not essential.

A risk assessment tool for contaminated sites in low-permeability fractured media
This report presents ecotoxicological data and Predicted No-Effect Concentrations (PNECs) for nine selected nanomaterials which are considered to be environmentally relevant due to high usage or how they are used. These data will together with data from other reports/projects be used in an overall assessment of the environmental risk of nanomaterials in Denmark.

The nine investigated nanomaterials are: Titanium Dioxide, Zinc Oxide, Silver, Carbon Nanotubes, Copper Oxide, Nano Zero Valent Iron, Cerium Dioxide, Quantum Dots and Carbon Black.

To support the assessment of the data found in the peer reviewed scientific literature, the current project has developed a scoring system that evaluates the liability and relevance of the data in relation to nanomaterials.

Nærværende rapport præsenterer økotoksikologiske data og Predicted No-Effect Concentrations (PNECs) for ni udvalgte nanomaterialer, som forventes at være miljømæssigt relevante ud fra viden om forbrugsmængder eller hvordan de anvendes. Disse data skal sammen med data fra andre rapporter/projekter bidrage til en samlet vurdering af nanomaterialers miljørisiko i Danmark.

De 9 undersøgte nanomaterialer er: Sølv, Titaniumdioxid, Zinkoxid, Kulstofnanorør, Kobberoksid, Nanojern i oxidationstrin nul, Ceriumdioxid, Carbon black samt Kvantepunkter.

Som en del af arbejdet med at finde data i litteraturen, er der i dette projekt udviklet et point-system til evaluering af data pålidelighed og relevans i forhold til nanomaterialer.

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